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Marnix Jansen and
Nicholas A. Wright *Editors*

Stem Cells, Pre-neoplasia, and Early Cancer of the Upper Gastrointestinal Tract

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Chapter 1
Distal Esophageal Adenocarcinoma and Gastric Adenocarcinoma: Time for a Shared Research Agenda

Marnix Jansen and Nicholas A. Wright

What we had for dinner today would sound very odd in England,
Charles Darwin (September 18, 1832)

It remains unclear when and where Charles Darwin had his transformative idea of species evolution through random variation and selective retention. The legend goes that the first inklings of the idea came to him on board the Beagle during the ship's visit to the Galapagos Islands in the Eastern Pacific. Here he beheld finches with differently shaped beaks, which suggested local adaptations to varying diets. However, there are clear indications that the idea came to him several years before. Three years before to be exact. Over dinner. Along the eastern coast of what is now Argentina.

The notebook entry above dates from the first year of the Beagle's voyage when the ship was anchored near Bahia Blanca, an outpost some 700 km south from Buenos Aires. The explorers ate whatever the hunters brought back from the fertile Argentine Pampas, mostly deer, agoutis, armadillos, and rhea (which the budding naturalist called "an ostrich"). At these sites Darwin and a helper set to work on the soft rock to uncover fossils. At the first site (Punta Alta) he recovered the remains of no less than nine great mammals. Most of these Pleistocene giant mammals are unique to the Americas, the most famous of which was a huge sloth called Megatherium, and were hardly known at the time to science. In this setting Darwin also recovered specimens of animals that reminded him of the armadillos that he had had for dinner (reputed to taste terrible).

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About a month later at Monte Hermoso he unearthed the fossilized remains of several gnawing creatures, which reminded him of a capybara, an agouti, and a tuco-tuco (a smaller rodent native to South America). In each case, however, the resemblance between fossil and living species was close, although not identical. At this time he also unearthed a complete set of bones, which suggested an extinct form of camel because it resembled the guanaco (the wild form of the llama). Again Darwin had shot one himself just days earlier.

All of these analogies, similar in form but not exact copies, between fossils that he had unearthed from the ground and those that he observed daily on the plains of the Argentinian Pampas (and on his dinner plate) fermented in Darwin's imagination. Meanwhile Darwin prepared the fossils for shipping back to England to his long time mentor John Stevens Henslow. In a letter to Henslow he wrote: "I have been lucky with fossil bones […]. Immediately I saw them I thought they must belong to an enormous Armadillo, living species of which genus are so abundant here." Ever the gentleman he added: "If it interests you sufficiently to unpack them, I shall be very curious to hear something about them."

Several months went past as the Beagle sailed further south along the coastline and finally reached Northern Patagonia (Fig. 1.1). This landscape is very different from the Pampas further up north. It is wild and rugged, mountains rise straight from the ocean and glaciers can be seen streaming down the Andes, which quite literally mold the landscape and carve out isolated green plains surrounded by almost insurmountable peaks. Darwin had been a geology student and it is not difficult to imagine how in this landscape he first witnessed what had till then seemed merely an intellectual abstraction: that over time landscapes slowly change and, sometimes quite abruptly, alter habitats for extant species. Here Darwin befriended a group of gauchos who told him of a rare form of "ostrich." This bird was apparently smaller (and easier to kill), but otherwise similar to the rhea, which lived further up north. One night his shipmates returned with a specimen, which they had caught for dinner. At first Darwin paid little attention, likely assuming the bird was a juvenile of the larger rhea and the animal was prepared for dinner as per usual. However, suddenly that evening it seems Darwin remembered the relationship

Fig. 1.1 Stunning landscapes of the Los Glaciares National Park close to El Calafate in Southern Argentina (private collection). Charles Darwin sailed up this river on the Beagle in 1834 collecting specimens for his research

with the larger rhea. In his notebook he recorded: "The bird was skinned and cooked before my memory returned, but the head, neck, legs, wings, many of the larger feathers, and a large part of the skin, had been preserved." He rescued whatever was left and prepared this for shipping to England.

It seems Darwin was particularly struck by the fact that although both species of rhea clearly resembled one another, they overlapped very little in geographic distribution. Darwin's observations on rhea diversity and distribution now finally started to blend with his earlier observations on morphologic relatedness of fossils and living animals, into a singular story. The sloths and armadillos seemed to have succeeded earlier such forms in time, but inhabiting roughly the same terrain during different geological chapters of Earth's history. By contrast, the two rheas, again similar but not identical, likewise seemed to succeed each other—but in this case the two species succeeded each other in space. This succession in time and space therefore suggested that these animals had descended, with modification, from common ancestors: smaller rheas from larger rheas, sloths from earlier sloths, and armadillos from an armadillo(-like) precursor.

From his vivid descriptions of the food onboard the Beagle, it is clear how Darwin's culinary experiences on the Pampas and in Patagonia played a tremendous part in his evolutionary thinking. And although the finches on the Galapagos Islands have taken much of the public credit for igniting the idea of descent with modification in Darwin's reasoning, it seems more likely that these ideas took root years before—the finches only cemented the idea by confirming the general principle. Indeed the first lines of "On The Origin Of Species" read: "*When on board H.M.S. 'Beagle,' as naturalist, I was much struck with certain facts in the distribution of the inhabitants of South America, and in the geological relations of the present to the past inhabitants of that continent. These facts seemed to me to throw some light on the origin of species*."

Research into the molecular evolution and approach to clinical management of early gastric and distal esophageal cancer has accelerated tremendously in recent years. Endoluminal resection and disease staging can now be considered standard of care for patients with early stage disease. In patients with neoplastic change of the distal esophagus, these excisions can be complemented (or in selected cases even supplanted) by ablative treatment [1, 2]. Likewise where several years previous we only had a superficial understanding of the spectrum of molecular "driver" events in early gastric and distal esophageal cancer evolution, we now know the culprit genetic alterations in great detail. Genomic profiling of gastric cancer has shown that gastric cancer can be subclassified in several core molecular subtypes and, importantly, these subtypes show vastly different clinical behaviors [3, 4]. Recent research in esophageal adenocarcinoma has shown that at least two progression pathways can be discerned; whether these molecular subtypes also show a different clinical behavior remains unclear [5, 6].

The definition of disease subtypes at these earlier stages may allow us to define molecular biomarkers, which facilitate prognostication and risk stratification of individual patients [7]. These biomarkers ideally guide tailored treatment, which may improve clinical care by avoiding overtreatment of patients with limited risk of progression while allowing intensive surveillance or more aggressive treatment in

those with greatest risk of progression. Biomarkers may also allow us to make rational choices and weigh therapeutic intervention against current clinical status and expected gain. These studies provide a basis for understanding progression pathways in patients struck by early esophageal or gastric cancer and made it clear that the heterogeneity in clinical disease behavior is reflected in the molecular underpinnings of these diseases.

Importantly, most of these studies have been performed in patients who present with late-stage disease, but this heterogeneity is seen across the disease spectrum, even at preneoplastic stages [8]. For example, some patients with extensive metaplastic changes never develop progressive lesions, whereas others present with lethal, bulky disease with minimal metaplastic background change. Fundamentally, we do not understand what underlies this clinical heterogeneity; however, slowly we are now beginning to discern disease subtypes in esophageal and gastric cancer progression.

This volume takes an interdisciplinary approach to the problem of clinical heterogeneity in upper GI cancer. We aimed to bring together leading authorities in the fields of clinical medicine and translational research of gastric and esophageal adenocarcinoma. We selected the topics of interest and recruited a series of authors based on recent publications that have particularly advanced our understanding of cancer progression in upper GI cancer. Where possible we aimed to include several (possibly opposing) viewpoints to further stimulate discussion.

The 22 chapters in this volume are divided into three sections: one section on esophageal adenocarcinoma, one section on gastric adenocarcinoma, and one introductory section detailing several basic concepts pertaining to both fields. We asked our authors to take this opportunity to write argumentatively about outstanding questions in their field as we felt that rather than collating a series of reviews on the current state of the field, it would be rather stimulating to discuss future directions.

Following this introduction *Juergen Fink and Bon-Kyoung Koo* recapitulate fascinating studies conducted in recent years on the clonal evolution of stem cell niches in the GI tract. These studies have shown us that stem cell niches in the GI tract demonstrate a stereotypic modular makeup. By studying the clonal evolution of individual stem cell niches we may able to understand the earliest steps toward GI cancer, which underscores the translational importance of their work. Mutations expand in the epithelium through the duplication of mutant stem cell niches. *James Evans and Stuart McDonald* discuss studies which have investigated this early ("pretumor") phase of cancer formation. Finally, *Parakrama Chandrasoma and Tom DeMeester* discuss our current understanding of the development of possibly the most contentious topic of all in upper GI cancer, the gastric cardia. Chandrasoma and DeMeester propose a novel and an attractive theory for the ontogeny of the gastric cardia, which states that the cardia is an esophageal "damage zone" which is remodeled over time from tubular esophagus to saccular proximal stomach.

Part 2 of this volume is on cancer progression in the distal esophagus. First, *Anne-Fré Swager, Wouter Curvers and Jacques Bergman* discuss the state of the art in the endoscopic detection of Barrett's neoplasia. Their guiding principles are as follows: always use your best endoscope available; perform a systematic endoscopic inspection; and lastly, "you do not detect what you see, you detect what you

recognize." Their manuscript on the detection of Barrett's neoplasia is followed by a paper by *Oliver Pech*, who discusses the extensive experience that has been gathered over the recent years in their impressive Wiesbaden cohort. Oliver Pech focuses specifically on the immediate need for biomarkers that will allow us to understand in greater detail which patients with deeper penetrating T1b lesions can be safely treated endoscopically. These twinned manuscripts on the endoscopic detection and treatment of early Barrett's neoplasia are followed by a pair of manuscripts on the histopathology of Barrett's esophagus and its dysplastic cancer precursor lesions: *Hannah Lowes, Thusitha Somarathna, and Neil Shepherd* discuss histopathological perspectives on the definition, derivation, and diagnosis of Barrett's esophagus, while *Myrtle van der Wel, Marnix Jansen, Michael Vieth, and Sybren Meijer* examine the histopathology of Barrett's dysplasia. The final clinically oriented manuscript in this part of the volume comes from *Oli Old, Martin Isabelle, and Hugh Barr.* These authors discuss surgical and oncological perspectives to the clinical staging of patients with esophageal cancer and focus on the exciting prospect of novel imaging techniques such as optical coherence tomography and Raman spectroscopy which may allow endoscopic staging in real time. We then focus on the development of Barrett's esophagus. Although many tissue sources have been proposed as stem cell compartments from which metaplastic Barrett's epithelium may originate (including the proximal stomach, the submucosal esophageal gland, the bone marrow, and even a niche of specialized embryonic precursor cells located at the gastro-esophageal junction), the exact origin remains unknown. *David Wang and Rhonda Souza* discuss recent data from their laboratory demonstrating how squamous epithelium—ill protected against the caustic impact of acid-biliary reflux—may switch to a mucin-producing columnar phenotype. *Tom Paulson* discusses studies from one of the longest running longitudinal cohorts investigating clonal evolution to cancer in Barrett's esophagus. Their cohort has provided many fascinating insights into the dynamics of cancer progression of Barrett's esophagus and this manuscript focuses on elegant studies, which have shown that, quite remarkably, genetic progression to cancer in Barrett's esophagus occurs within a relatively narrow timeframe of only 3–4 years. His manuscript also deals with some of the problems in setting up these large-scale studies. *Mark Pusung, Sebastian Zeki, and Rebecca Fitzgerald* discuss the genomics of cancer progression in the distal esophagus. The Fitzgerald laboratory is one of the leading working groups in deciphering the genomic driver events that provoke cancer progression in the distal esophagus. This important chapter provides a broad overview of their work and others and also addresses the search for predictive clinical biomarkers in Barrett's esophagus. Finally, *Claire Palles, John M Findlay, and Ian Tomlinson* recapitulate their work on germline variants that mediate susceptibility to the development of Barrett's esophagus. Their studies have provided fascinating data showing that susceptibility to the development of Barrett's esophagus is linked with variants in patterning genes associated with esophageal development (such as BARX1 and TBX5) and with variants in the major histocompatibility complex suggesting that response to injury may similarly play a role.

Part 3 of this volume on cancer progression in the stomach in many ways mirrors the layout of part 2 on cancer progression in the distal esophagus. First, *Noriya Uedo and Kenshi Yao* discuss the endoscopic detection of early gastric cancer and its precursors. This chapter is followed by work from *Ichiro Oda, Harushisa Suzuki, and Shigetaka Yoshinaga* who discuss their extensive experience in the endoluminal treatment of early gastric cancer through endoscopic submucosal dissection. Japan has long been a frontrunner in the development of endoscopic treatment algorithms of early gastric (and esophageal) neoplasia. These manuscripts are lavished with beautiful illustrations (and some videos), which capture essential diagnostic criteria. These chapters on the endoscopic detection and treatment of early gastric cancer are followed by a chapter by *Shigeki Sekine, Hiroshi Yoshida, Marnix Jansen, and Ryoji Kushima* on the histopathology of early gastric cancer. There is a great deal of debate (and confusion) surrounding the issue of early invasion in the neoplastic stomach between Western and Japanese histopathologists. Staging systems that were originally meant to bridge these differences (such as the Vienna system) in retrospect appear to have only cemented the idea that the differences are irreconcilable. This chapter aims to tackle this issue by underscoring the commonalities and the need for further research into (objective molecular) biomarkers. The following two chapters focus on cancer progression in familial context. *Lodewijk Brosens, Frank Giardiello, Johan Offerhaus, and Elizabeth A. Montgomery* discuss the histopathology of precursor lesions which may be found in patients at increased risk for gastric adenocarcinoma. Interestingly, development of these lesions can be accelerated by environmental risk factors, which elegantly illustrates how genetic background and environment interact to mediate cancer risk. Next, *Rachel van der Post, Irene Gullo, Carla Oliveira, Laura Tang, Heike Grabsch, Maria O'Donovan, Rebecca Fitzgerald, Han van Krieken, and Fátima Carneiro* discuss the latest research on hereditary diffuse gastric cancer. The exact mode and tempo of the progression of diffuse gastric adenocarcinoma remains very much unclear. Importantly, these authors present important original data, which may help risk stratify early lesions in these patients. In future this may help us understand differences in diffuse gastric cancer progression in familial versus sporadic patients. We then turn our attention on the gastric microbiome. *Lydia Wroblewski and Richard Peek* provide us with an overview of fascinating recent data indicating that alterations to the composition of the gastric microbiota following Helicobacter infection may determine the risk of progressing the gastric neoplasia. This chapter is followed by clinically oriented chapter in which *Marino Venerito, Riccardo Vasapolli, and Peter Malfertheiner* discuss our current understanding of the impact of Helicobacter eradication, in particular with regards to the timing of eradication. Lastly, we focus on the genetics and genomics of gastric adenocarcinoma. *Siu Tsan Yuen and Suet Yi Leung* discuss elegant data from their laboratory which has investigated the genomics of a large cohort of gastric adenocarcinoma patients. Their results indicate that gastric adenocarcinoma can be subclassified into several molecular subtypes. Importantly, this classification is also predictive of clinical outcome. Finally, *Lin Ding, Mohamad*

El Zaatari, and Juanita Merchant provide us with a broad overview of studies that have been conducted toward developing animal models, which reflect gastric cancer initiation and pathogenesis. These models provide us with excellent systems to test the impact of genetic alterations on cancer progression as well as the effect of treatment strategies.

Ultimately, with the tools now at our disposal to dissect esophageal and gastric cancer evolution in great detail the time is now to ask the right questions. The majority of patients struck by upper GI cancer are still diagnosed at incurable stages. It is hoped that our increased understanding of the genetics and genomics of upper GI cancer progression will translate to earlier diagnosis and greater survival. For those patients who are diagnosed at earlier stages, we must now couple the availability of elegant endoscopic imaging and treatment techniques to effective and objective clinical biomarkers, which predict aggressive disease and a poor outcome in patients with limited disease such that these patients may be offered further treatment while others are spared such aggressive treatment. Finally, and most tantalizingly, our understanding of the early, precursor stages should materialize into clinical biomarkers, which predict disease progression before neoplastic derailment.

These problems are not unique as they are also faced by researchers working on other (gastrointestinal) cancers [9, 10]. However, one ace up our sleeve is the fact that gastric and esophageal adenocarcinoma share many histopathological and genetic characteristics of disease progression, morphologically as well as genetically. The key insight that sparked Darwin's theory of descent with modification was that he compared and contrasted differences between species across time and space. We feel that further integration of research into gastric and esophageal adenocarcinoma may benefit both fields. For example, we lack a complete understanding of the cellular and genetic processes leading to intestinal metaplasia. Although the environmental trigger differs between intestinal metaplasia of the stomach and distal esophagus, we feel that there are many important lessons still to be learned from comparing these precursor stages. The grand challenge moving forward will be to connect evolutionary changes in (cancer) genomes with particular evolutionary changes in phenotypes, and from this analysis to determine which phenotypic and functional alterations are driven by selection. This analysis will absolutely require detailed sampling across and between species (esophageal and gastric adenocarcinoma) and across time and space. Most importantly, we need higher resolution clinical phenotyping to relate genomic differences to morphologic evolution. In the end, this may provide us with a new phylogeny showing key differences between esophageal and gastric adenocarcinoma which will supersede previous classifications.

We are deeply indebted to all contributing authors for their time and energy in creating this collection of superb articles with figures and tables. We hope that readers of this volume will find new insights and enjoy the approach of this work and we anticipate that further interdisciplinary research will allow us to accelerate translation of research findings "across anatomic borders."

References

1. Shaheen NJ, et al. Radiofrequency ablation in Barrett's esophagus with dysplasia. N Engl J Med. 2009;360:2277–88.
2. Pouw RE, et al. Efficacy of radiofrequency ablation combined with endoscopic resection for Barrett's esophagus with early neoplasia. Clin Gastroenterol Hepatol. 2010;8:23–9.
3. Network TCGAR. Comprehensive molecular characterization of gastric adenocarcinoma-supplementary materials. Nature. 2014;513:202–9.
4. Cristescu R et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med 2015;21:449–56.
5. Stachler MD, et al. Paired exome analysis of Barrett's esophagus and adenocarcinoma. Nat Genet. 2015;47:1047–55.
6. Ross-Innes CS, et al. Whole-genome sequencing provides new insights into the clonal architecture of Barrett's esophagus and esophageal adenocarcinoma. Nat Genet. 2015;47:1038–46.
7. Yeh JM, Hur C, Ward Z, Schrag D, Goldie SJ. Gastric adenocarcinoma screening and prevention in the era of new biomarker and endoscopic technologies: a cost-effectiveness analysis. Gut. 2015. doi:10.1136/gutjnl-2014-308588.
8. El-Zimaity HMT, Ota H, Graham DY, Akamatsu T, Katsuyama T. Patterns of gastric atrophy in intestinal type gastric carcinoma. Cancer. 2002;94:1428–36.
9. Iyer G, et al. Genome sequencing identifies a basis for everolimus sensitivity. Science. 2012. doi:10.1126/science.1226344.
10. Waddell N, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. Nature. 2015;518:495–501.

Part I
Introduction

Chapter 2
Clonal Evolution of Stem Cells in the Gastrointestinal Tract

Juergen Fink and Bon-Kyoung Koo

The intestine is one of the fastest renewing tissues in our body. Its epithelial layer renews every 3–5 days in the adult. This rapid turnover is sustained by a small number of tissue-specific stem cells. Previous studies aimed to identify a rare population of long-lived cells by conventional means such as electron microscopy, DNA label retention, and staining with several rare cell markers [1–5]. Nevertheless, these studies were not sufficient to definitively identify the true potential of these adult stem cells in tissue homeostasis and regeneration. In 2007, Barker et al. reidentified crypt base columnar (CBC) cells as the stem cell of the intestine using elegant lineage tracing of $Lgr5^+$ cells [6]. These $Lgr5^+$ cells proliferate every day to produce a sufficient number of progenitors to fill up the pocket-like structures known as crypts (Fig. 2.1). Proliferating progenitors migrate upward while differentiating into nutrient-absorbing enterocytes as well as secretory cells that produce mucins (goblet cells) or hormones (enteroendocrine cells). These three cell types comprise the epithelium of the villus, a digit-like protrusion toward the gut lumen. Paneth cells migrate downward and stay together with CBC cells. They play a key role in the secretion of antibacterial compounds and in stem cell maintenance by providing growth factor signals (e.g., Egf, Notch, and Wnt ligands) [7].

Lineage tracing experiments have become the gold standard for investigating the longevity and differentiation potential of adult stem cells in vivo. Currently, lineage tracing experiments mostly rely on the tamoxifen-inducible Cre-Recombinase enzyme and transgenic reporter mice (e.g., Rosa26-reporter) [8–18]. In this system the Cre DNA recombinase is linked to a fragment of the estrogen receptor (ER), generating a fusion protein called CreER [19]. The Cre activity of this artificial

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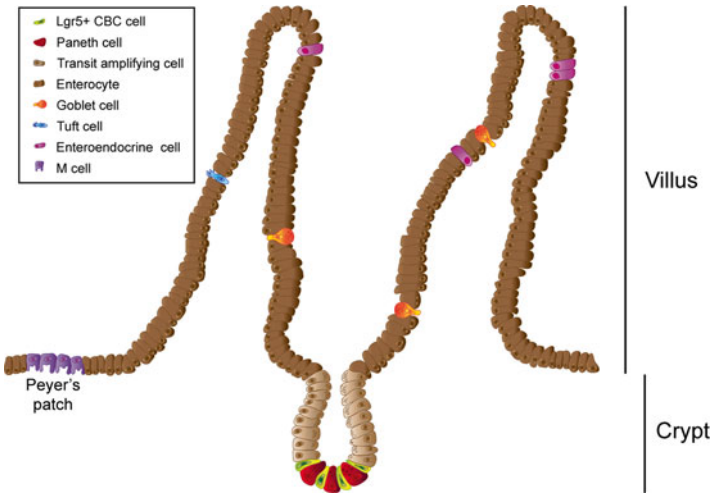

Fig. 2.1 Epithelial layer of the small intestine (adapted from Koo et al. [51]). The epithelial barrier of the small intestine is comprised of a single cell layer that forms protrusions, called villi, and invaginations, called crypts. Crypt base columnar (CBC) cells can be found in the crypt base intermingled with Paneth cells. Stem cell proliferation drives cell migration upward to replace the functional cell types within the intestinal epithelium. CBC cells mainly generate transit amplifying cells that are located just above the crypt base. During their upward migration these cells proliferate and differentiate to nutrient absorbing enterocytes, secretory enteroendocrine, or goblet cells. Microfold cells (M cells), involved in antigen presentation to the immune system, and tuft cells of unknown function are also generated during this process. Paneth cells remain in the crypt base to provide crucial niche factors for the CBC stem cell population

fusion protein is often under spatial control as it can be placed under a tissue-specific promoter. Its activity can also be regulated temporally as it enters the nucleus only if it binds to its ligand estrogen or the synthetic analog tamoxifen, which can be provided via a direct intraperitoneal injection to the mouse. Once it moves into the nucleus, CreER can facilitate recombination between LoxP sites. When combined with a Cre reporter, tamoxifen-activated CreER can excise a LoxP-flanked "stopper" cassette of the reporter to induce the expression of β-galactosidase or a fluorescent protein. Placing the CreER enzyme under the control of a stem cell gene-specific promoter, this enzyme can permanently mark a stem cell by genetically removing the stopper. Once activated, this genetic change is inherited by all progeny from the marked stem cell (Fig. 2.2).

As explained, the heart of this method lies in the identification of a specific marker gene of the target cell type [20]. The stem cell nature of CBC cells was not well understood until the Wnt target gene Lgr5 was identified as a specific stem cell marker. Employing this novel marker, lineage tracing experiments proved CBC cells are long-term adult stem cells in the gut epithelium [6]. Technically, an Lgr5-eGFP-ires-CreERT2 knock-in mouse was designed to express both eGFP and CreERT2 simultaneously under the control of the endogenous Lgr5 promoter. In this way, the

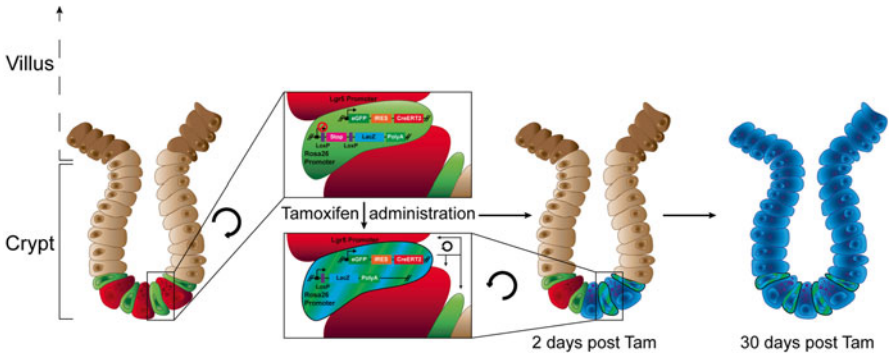

Fig. 2.2 Lineage tracing in Lgr5⁺ CBC cells of the small intestine. In a mouse model expressing eGFP and the tamoxifen-inducible CreERT2 enzyme under the transcriptional control of the Lgr5 promoter, all Lgr5⁺ cells express GFP and CreERT2. Upon tamoxifen administration the Cre recombinase relocates into the nucleus. In combination with reporter alleles (e.g., RosaR26-LacZ), the recombinase induces the expression of the reporter gene by excision of a stop signal. Subsequently, Lgr5⁺ CBC cells are labeled both with Lgr5-promoter-controlled eGFP and with the constitutively expressed reporter gene. Labeled stem cells self-renew and generate functional cell types of the intestinal epithelium without losing the reporter label, resulting in complete labeling of the entire crypt-villus axis

CBC cells could be visualized by eGFP and they also expressed tamoxifen-inducible CreERT2 in a stem cell-specific manner [6]. By administering tamoxifen to Lgr5-eGFP-ires-CreERT2;Rosa26-reporter mice, a single Lgr5⁺ CBC cell could be labeled to express a reporter gene (e.g., β-galactosidase) under the control of the constitutively active Rosa26 promoter. One day after the induction, X-gal staining revealed specific induction of β-galactosidase in cells at the crypt base. Within 5 days, the progeny from this single cell formed a longitudinal blue ribbon in the epithelium, suggesting that all cells within the ribbon are derived from a single Lgr5⁺ cell [6] (see also Fig. 2.2). Immunohistochemical analysis revealed the existence of all known intestinal epithelial cell lineages of the intestine in this ribbon [6, 21, 22]. Most importantly, these ribbon-shaped whole crypt-villus axis tracings were readily detectable at time points of more than 1 year posttamoxifen injection, proving that Lgr5⁺ CBC cells indeed represent a long-lived adult stem cell population of the intestinal epithelium [6].

Lgr5 marks not only intestinal stem cells but also stem cells in the pylorus glands of the stomach and in colonic crypts [6, 23]. Other genes that are specifically expressed in CBC cells are Tnfrsf19 (Troy) as well as Olfm4, Ascl2, and Smoc2 [24]. Troy lineage tracing experiments revealed a slowly cycling stem cell population in the gastric corpus glands [25]. With these two markers, we have now identified many endodermal adult stem cells from stomach to colon. In this book chapter, we will first describe clonal behavior of intestinal stem cells in homeostasis, regeneration, and tumorigenic alteration. We will then summarize our recent understanding of the clonal behavior of gastric stem cells. Finally, the relationship between novel Troy⁺ corpus stem cells and conventional gastric isthmus stem cells will be discussed.

How Many Stem Cells Are in the Intestinal Crypt?

The number of stem cells needs to be strictly regulated to avoid the generation of too many transit amplifying or differentiated cells within the intestinal epithelium. Lgr5$^+$ CBC cells divide once a day, generating new CBC cells which reside at the base of each gland as stem cells [26]. The location of these CBC cells deep within the pocket-like crypts is key to the tight control of the stem cell population. As there is limited space for stem cells in the crypt base, only a fixed number of stem cells can fit into this stem cell zone. Consequently, each stem cell clone competes for this limited space with other stem cell progeny and only the winning stem cell clone of this competition can occupy the whole crypt with its clonal descendants. As all stem cells initially have the same chance to be the winner, this process is described as "neutral competition." Under homeostatic conditions, the winning stem cell clone in the crypt base gives rise to all the functional cell types of the intestinal epithelium. The cellular hierarchy under these conditions is strictly regulated, so that cells that are pushed out of the stem cell niche are first committed to a transit amplifying progenitor fate before differentiating to the various terminally differentiated cell types (e.g., enterocytes, goblet cells, enteroendocrine cells, and Paneth cells).

So how many stem cells are in the crypt? Initially, the number of stem cells was deduced from the number of Lgr5$^+$ stem cells in the crypt. Using flow cytometric analysis of Lgr5$^+$ intestinal stem cells, Snippert et al. [26] carefully set the threshold of Lgr5-GFP intensity that determines a defined population of Lgr5$^+$ cells in their flow cytometry data. Based on this GFP intensity level, the authors counted the number of Lgr5-GFP$^+$ cells in the crypts of the duodenum, jejunum, and ileum. On average, each small intestinal crypt contains around fourteen Lgr5$^+$ cells. Only crypts from the ileum displayed slightly higher numbers. In this case, the authors regarded all Lgr5$^+$ cells as functional stem cells in the crypt, since Lgr5$^+$ stem cells were found to be a single population coexpressing various other stem cell markers (e.g., Olfm4, Ascl2, and Smoc2). However, the effect of crypt structure and the limited niche space were not fully taken into consideration in this analysis. A few years later, another group developed a novel strategy—continuous clonal labeling. Using this method, they noticed increasing numbers of fully labeled crypts as well as stable fractions of partly labeled crypts. With a given mutation rate, the group predicted the actual number of working stem cells in the crypt to be only 5–6 in the small intestine [27], which is much fewer than initially predicted [26]. Despite accurate modeling of stem cell behavior, all approaches that aimed at understanding how stem cell populations compete with each other are based on the analysis of multiple independent stem cell clones at various time points and the retrospective development of models that can fit the observed clonal expansion data. To catch a glimpse of the actual clonal competition within the intestinal crypts, the group of van Rheenen established a sophisticated in vivo live-imaging technology to study intestinal stem cell behavior. This revealed heterogeneous clonal behavior of Lgr5$^+$ cells, with cells located close to the center of the crypt having a much higher probability of generating a clone that could occupy the entire crypt, suggesting that

the physical position of each stem cell could be a major determinant of stem cell potential, even if each stem cell has the same biological properties [28].

What Factors Determine Stem Cell Number in the Crypt?

It is thought that mainly Paneth cells and underlying mesenchymal cells are responsible for the niche formation crucial for stem cell maintenance and regulation. Both Paneth cells and the underlying, yet unidentified, mesenchymal cells produce the most potent growth factor of the intestinal epithelium—Wnt ligands. These cells also produce other growth factors supporting Lgr5$^+$ stem cells. Currently, the Paneth cell is the best characterized support cell for Lgr5$^+$ stem cells. Paneth cells reside together with Lgr5$^+$ stem cells at the crypt base (Fig. 2.3a). The basal membrane of Paneth and stem cells shows icosidodecahedron-like geometry, where the large Paneth cells occupy pentagons and the small Lgr5$^+$ stem cells are squeezed in between the Paneth cells as triangles. This structure maximizes the shared membrane between Lgr5$^+$ stem cells and Paneth cells while minimizing isologous interactions, suggesting the importance of the heterologous interaction between stem cells and Paneth cells [7, 26]. Indeed, Paneth cells provide growth factor signals such as EGF, Wnt3, and Notch ligands [7], which have been shown to be crucial for stem cell maintenance both in vivo and in vitro.

Paneth cells have been identified to at least partially provide the niche required for stem cell regulation. Depletion of Paneth cells via loss of Sox9 resulted in Olfm4$^+$ CBC stem cell loss, illustrating the close functional relationship between Paneth cells and intestinal stem cells. Paneth and other secretory lineage precursors in the crypt express the Notch ligands Dll4 and Dll1, respectively. Removal of both ligands caused stem cell exhaustion [29]. Ablation of Wnt3 from the intestinal epithelium failed to show its importance in stem cell maintenance in vivo due to redundant Wnt ligands being secreted by the underlying mesenchyme [30]. Nevertheless, the indispensable role of Wnt3 for stem cell maintenance has been shown in Wnt3-null intestinal organoids, which were unable to survive in vitro as the organoid stem cell population, lacking alternative Wnt sources, is fully dependent on the paracrine Wnt source provided by Paneth cells [30]. Interestingly, Math1 mutation in the intestinal epithelium liberates Lgr5$^+$ stem cells from their requirement for Paneth cells and Notch activation [30–32]. However, in vitro organoid culture of Math1 mutants again proved the importance of Paneth cells, the paracrine Wnt source for intestinal stem cell maintenance [30, 32]. An accurate management of stem cells by Paneth cells was reported in normal and fasting status [33]. Calorie restriction leads to attenuation of the mTORC1 signaling pathway in Paneth cells resulting in production of cyclic ADP ribose, which in turn stimulates the self-renewal of intestinal stem cells. Taken together, while a stromal niche has an influential effect on intestinal stem cell maintenance, the Paneth cell is a major player in the generation of the epithelial niche, which delicately controls the behavior of stem cells through their close contact.

Neutral Competition and the Rules of the Game

As described earlier, in neutral competition stem cells compete for the limited niche space within the crypt. Stem cell-derived clones can undergo (1) expansion, (2) contraction, or (3) irreversible extinction (Fig. 2.3b) [34, 35]. Although every stem cell is predicted to have the same potential to win this competition, we now have evidence that stem cells residing close to the center have a higher chance of being the winner. Under homeostatic conditions, we postulate that this neutral competition among stem cells is largely affected by the physical environment rather than by biological differences among individual stem cells. For instance, the proximity to the center of the crypt base, contact area between stem cells and Paneth cells, and the strength of

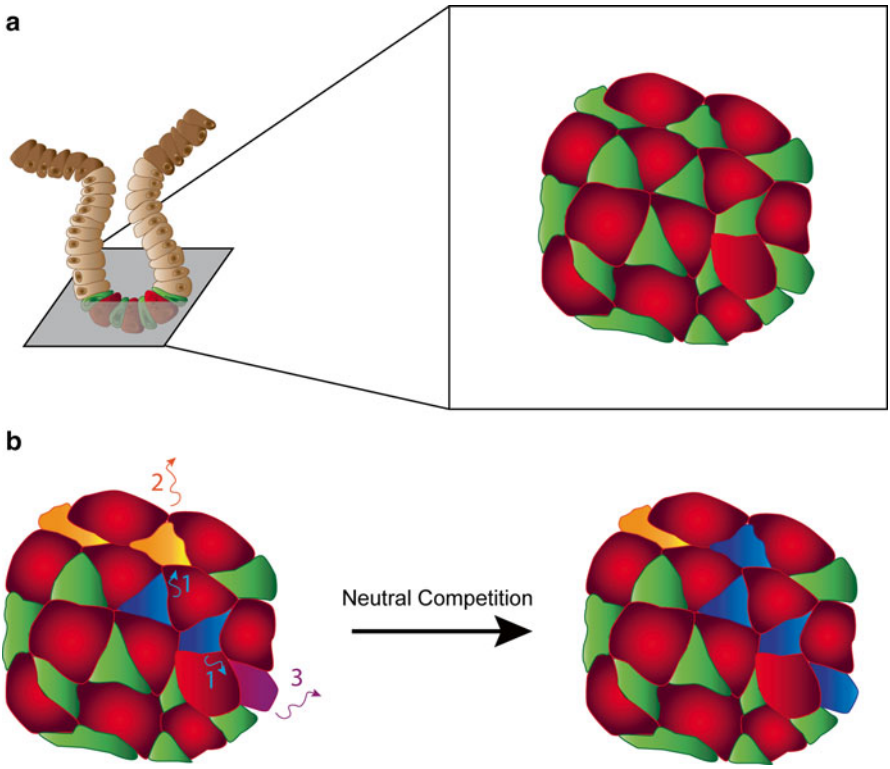

Fig. 2.3 Neutral competition illustration in intestinal crypts. Stem cells (*green*) of the small intestine are intermingled with Paneth cells (*red*) at the base of intestinal crypts (**a**). Clonal labeling illustrates the possible neutral competition-mediated outcomes for each clone (1): Expansion; (2): Contraction; (3): Extinction (**b**). The blue clone is located in the center of the crypt base tightly associated with Paneth cells whereas the orange and the purple clone are located toward the edge of the stem cell zone. Clonal expansion (1) of the blue clone results in Reduction (2) of the orange clone and Extinction (3) of the purple clone [28]

attachment to the basal matrix determine the chance of a stem cell staying within the stem cell zone. Thus, a stem cell located more centrally, with a larger area of contact with Paneth cells and basal matrix has a physically firm location that eventually proves advantageous to the stem cell toward being the victor of neutral competition (see Fig. 2.3b). However, all these physical conditions change in a dynamic manner such that a clone can only be the sole victor if it fills all stem cell niches in the crypt with its own daughter stem cells. It is important to keep in mind that the beginning of this neutral competition between stem cells is arbitrarily defined by the time point at which lineage tracing is induced. Clonal competition has occurred before this labeling event and will still continue after one clone has taken over the entire crypt.

In the neutral competition model it is assumed that all players are equally competent. However, in the actual biological context, this fair play can be biased by genetic alterations in each stem cell player. For example, tumorigenic mutations can provide a clonal advantage to stem cells. Both APC loss and K-Ras activation improve the clonal survival rate during clone competition [36, 37]. Interestingly, p53 mutation provides a similar clonal advantage only in specific contexts, such as inflammatory colitis. In other words, a stem cell having tumorigenic mutations has an advantage in filling up a whole crypt with its own daughter cells that carry the same mutation. As K-Ras activation and p53 loss alone do not cause an obvious morphological change, it is possible to accumulate these phenotypically invisible mutant cells with genetic lesions in crypts under homeostatic (K-Ras mutation) and inflammatory (p53 mutation) conditions.

What about stem cell players that are in danger of losing, or that are already out of, the competition? It is not the end for these "losers." Under specific conditions, certain cell types were shown to reacquire stem cell properties. Two special cell types have been identified by two groups: $Lgr5^+$ label-retaining cells [38] and $Dll1^+$ secretory progenitors [39]. Both cell types are not proliferative, or less proliferative than $Lgr5^+$ stem cells or other fast-dividing transit amplifying cells. These cells are committed early progenitors for the secretory lineages. Thus, they will mainly differentiate into terminally differentiated cells such as goblet cells, enteroendocrine cells, and Paneth cells. When the mouse intestinal stem cell compartment is perturbed by sublethal irradiation, rapidly dividing $Lgr5^+$ stem cells die and are quickly depleted from the stem cell zone. In this situation, both $Lgr5^+$ label-retaining cells and $Dll1^+$ secretory precursor cells can enter the stem cell zone, restore close contact with niche cells, and undergo dedifferentiation to regain stemness. Therefore, even after losing the game, an early committed progenitor can still rejoin the clonal competition as a stem cell.

These findings demonstrate not only the cellular plasticity of lineage restricted cells under tissue regeneration, but they also illustrate the importance of niche space-mediated stem cell maintenance. In this context, the limiting factor is again the number of Paneth cells providing niche space for the intermingled $Lgr5^+$ stem cell population. When all $Lgr5^+$ stem cells are depleted by γ-irradiation, a committed progenitor can enter into close contact with Paneth cells again. Niche factors from this Paneth cell are thought to help the committed progenitor to reacquire stem cell properties. Among other factors, the Wnt ligand was first found to be an important

niche factor that can allow Dll1$^+$ secretory progenitors to revert back to a stem cell fate, as sorted Dll1$^+$ cells were able to generate intestinal organoids containing Lgr5$^+$ cells if they were cultured in Wnt3a-containing media [39].

Taken together, the number of stem cells and clone dynamics in the stem cell zone of the crypt are believed to be tightly regulated by Paneth cells providing the niche space. Under normal homeostatic conditions, all stem cell players compete with the same chance to be the winner, yet the physical environment around each stem cell can affect the survival chances of individual stem cell clones. Lastly, a tumorigenic mutation can endow a significant clonal advantage to the mutant stem cell, whereas damage-induced stem cell loss may recall the losers of this competition (e.g., committed secretory progenitors) to play again as a dedifferentiated stem cell player in the game of neutral competition.

Dynamics in the Pyloric Glands of the Stomach Epithelium

The mouse stomach can be subdivided into three distinct zones. While the forestomach is comprised of a stratified epithelium, the corpus and the pylorus display a glandular epithelial organization (Fig. 2.4). Both pyloric and corpus glands can be subdivided into distinct zones: (1) pit, (2) isthmus, (3) neck, and (4) base. While glands of the pylorus consist primarily of mucous secreting cells, corpus glands show a distinct cellular composition in each of the four zones. The uppermost segment, the pit, contains mucus-secreting pit cells. The adjacent isthmus zone contains proliferative, undifferentiated cells. The next segment, the neck, is composed of mucous-secreting neck cells. The base is populated by pepsinogen-secreting chief cells. Hydrochloric acid-secreting parietal cells and hormone-secreting enteroendocrine cells are scattered throughout entire glands [40]. The highly specialized cell types that comprise the majority of pyloric and corpus glands have to be constantly replenished in order to maintain tissue function. This demand for differentiated cells requires a tightly controlled stem cell compartment at the base of the epithelial hierarchy, as previously described for Lgr5$^+$ stem cells of the intestine. Although pylorus and corpus glands are derived from the same embryonic origin, adult tissue homeostasis of the two regions appears to be differentially controlled.

For both pylorus and corpus, undifferentiated cells located in the isthmus have been proposed to represent a multipotent stem cell population that gives rise to all cell lineages of the adult epithelium [41–47]. In 2002, Bjerkens and Cheng applied a chemical mutagenesis-induced lineage tracing strategy to show that the adult gastric epithelium harbors functional multipotent stem cells [48]. In this approach, mice expressing β-galactosidase (LacZ) under control of the Rosa26 promoter are treated with the chemical mutagen *N*-ethyl-*N*-nitrosourea, resulting in mutation-mediated inactivation of the LacZ allele in some cells. If progenitor or multipotent stem cells are labeled by this approach, all their descendants will inherit the nonfunctional allele and will therefore not be labeled following LacZ staining. The group found evidence for the existence of long-term, self-renewing stem or

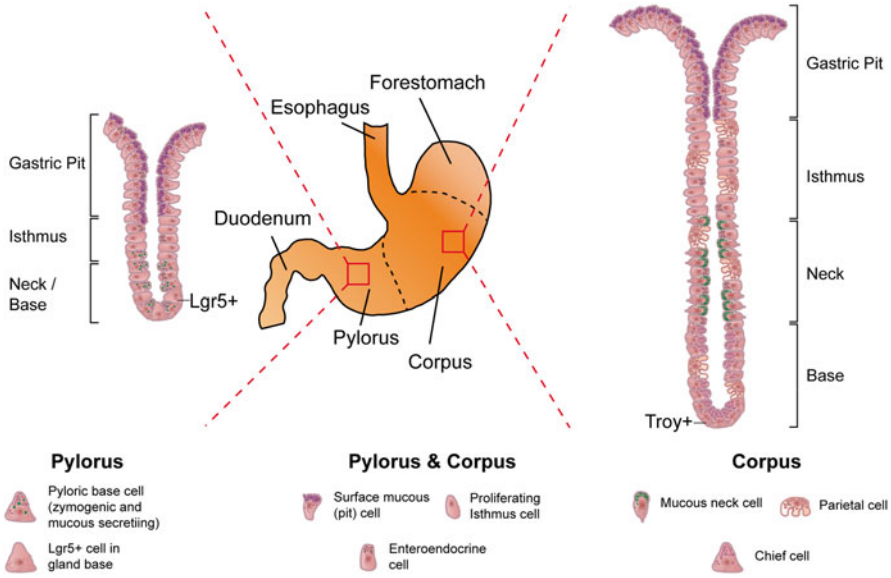

Fig. 2.4 Stomach structure and epithelial organization of the glandular corpus and pylorus. The mammalian stomach is divided into three parts: stratified forestomach and glandular corpus and pylorus. The glandular part shares a common organization, with glands being subdivided into four zones. Directly adjacent to the stomach lumen is the gastric pit, comprised of mucous pit cells. The cellular composition of the gastric pit is similar in corpus and in pylorus with the main function being secretion of mucous and subsequent protection of the stomach epithelium. Further within the gland is the isthmus zone, which harbors proliferative, granule-free, undifferentiated cells. In the corpus, at the bottom of the gland is the neck and the base, two distinct zones comprised mainly of mucous-secreting neck cells and zymogenic chief cells, respectively. In the pylorus, the zone at the base of the gland is comprised of cells that share mucous secreting and zymogenic features. Hormone-secreting enteroendocrine cells can be found in both corpus and pylorus, whereas parietal cells, responsible for the production of hydrochloric acid, are only found in the corpus. While in the pylorus $Lgr5^+$ stem cells in the gland base have been identified to be responsible for long-term tissue maintenance, in the corpus the isthmus is believed to harbor the long-term self-renewing stem cell population. $Troy^+$ chief cells, located at the corpus gland base, have been shown to respond to injury with rapid proliferation in order to regenerate the gastric epithelium

progenitor cell populations in the adult gastrointestinal epithelium. However, the exact position and identity of the tissue stem cells governing the homeostatic turnover of the entire gland were still unclear. The development of more sophisticated lineage tracing strategies using a putative marker for the pyloric stem cell was necessary to address this problem.

In 2010, the group of Hans Clevers was able to show that in the pylorus of the adult stomach, $Lgr5^+$ stem cells, residing at the bottom of gastric units, are responsible for long-term maintenance of the epithelium [23]. Long-term lineage tracing experiments revealed that $Lgr5^+$ stem cells are responsible for the homeostatic tissue turnover of the pylorus. In these experiments the clone size of $Lgr5^+$ stem cell-derived progeny was analyzed at various time points after labeling. Directly (2d)

after induction of lineage tracing, only Lgr5$^+$ cells in the base of glands are labeled. These labeled clones expand in the following days and result in fully labeled gastric units 10 days postinduction, highlighting the role of Lgr5$^+$ stem cells in short-term tissue homeostasis. Samples were taken up to 620d after the induction of lineage tracing and fully labeled gastric units could be readily observed, so proving the long-term self-renewal potential of the Lgr5$^+$ stem cell population in the pylorus. The combination of traditional lineage tracing strategies with mathematical modeling approaches allowed scientists to generate hypotheses about the exact mechanism of adult stem cell-mediated tissue homeostasis. As with Lgr5$^+$ cells in the small intestine, the Lgr5$^+$ stem cell population of the pylorus follows a neutral competition model for long-term self-renewal of the Lgr5$^+$ population rather than stringent asymmetric cell division-mediated self-renewal of each individual stem cell [49]. In this model, Lgr5$^+$ pyloric stem cells constantly self-renew and excess numbers of stem cells compete for the restricted niche space at the base of the glands. Consequently, over time, only one clone can survive and occupy the entire niche space of an individual gland. At this point this stem cell clone will occupy the entire axis of the gland with its own progeny. However, due to the joint restrictions of niche size and gland structure, the total number of stem cells does not increase indefinitely.

Interestingly, single Lgr5-expressing cells can form long-lived gastric organoids when plated into a 3-dimensional matrix and cultured in medium containing EGF, Noggin, R-spondin1, Wnt, and Fgf10. Under these culture conditions the Lgr5$^+$ stem cell population maintains its self-renewal potential and can generate various cell types of the gastric epithelium as shown by the expression of marker genes for chief cells and mucous neck cells (Gastric Intrinsic Factor, Pepsinogen-C, or Muc6). Slight changes of the culture conditions can direct differentiation toward Muc5ac-expressing pit cells, Periodic Acid Shift (PAS)- and Tff2-expressing mucous neck cells, and immature Chromogranin A-expressing enteroendocrine cells, suggesting that the cultured pyloric Lgr5$^+$ stem cell retains its multipotency as well as its self-renewal activity. In terms of population dynamics, this culture system suggested an intriguing aspect of the adult pyloric Lgr5$^+$ stem cells, in that these stem cells can self-renew indefinitely, albeit in vitro, when there is no restriction of niche components. This suggests that pyloric stem cells, as well as other gut stem cells from the intestine and colon, have no intrinsic limit to the number of cell cycles. Moreover, the culture conditions (e.g., basement matrix and growth factors) define the absolute niche requirement for this type of stem cells. Harnessing this unlimited self-renewal activity of adult stem cells will enable us to cultivate a large amount of adult stem cells for future cell-based therapy.

Clone Behavior in Corpus Glands of Stomach Epithelia

Despite the shared embryonic origin of the corpus and pylorus, Lgr5$^+$ stem cells could be found in the corpus only up until early postnatal stages and so appear to play no significant role during homeostasis of the adult tissue [23]. As described earlier and

similar to the pylorus, the gastric units of the corpus can be divided into pit, isthmus, neck, and base regions. Nevertheless, in contrast to the pylorus, the neck region of corpus gastric units displays a very different cellular composition, with multiple parietal cells and mucous neck cells physically separating the Isthmus from the base. Additionally, cycling cells can only rarely be found in the base of corpus glands, whereas Lgr5$^+$ cycling cells are a common feature of gastric units in the pylorus. Early labeling studies in combination with the description of cycling, immature cells located in the Isthmus lead to the assumption that the stem cell population responsible for tissue homeostasis of the corpus is located in the Isthmus zone [44–46, 48]. Nevertheless, the lack of a definitive marker of this putative stem cell population and the limitations of the chosen tracing strategies have hindered the exact identification and the conclusive proof of long-term self-renewing, multipotent stem cell populations.

In 2013, Stange et al. identified Tumor Necrosis Factor Receptor Superfamily Member 19 (Tnfrsf19 or Troy) as a potential marker that closely follows the expression pattern of Lgr5 in the small intestine [25]. In the corpus of the stomach, but not in the pylorus, Troy$^+$ cells have been identified in the base of gastric units. In this location Troy$^+$ cells were shown to be either chief or parietal cells. Lineage tracing analysis of both Troy$^+$ and chief cells revealed that Troy$^+$ chief cells but not Troy$^+$ parietal cells possess the ability to slowly repopulate entire glands, so highlighting their role as a reserve stem cell population. Labeled clones were shown to consist of all the epithelial cell types found in the corpus, illustrating the differentiation potential of Troy$^+$ stem cells. Additionally, labeled glands persisted in the epithelium for at least 1.5 years after the induction of lineage tracing, clearly illustrating the long-term self-renewing characteristics of this newly identified reserve stem cell population.

This study further emphasized how certain "fully differentiated" cells have a higher cellular plasticity than originally assumed (see also previously discussed label-retaining Lgr5$^+$ or Dll1$^+$ secretory precursor cells). Troy$^+$ stem cells share their primary role with other chief cells—pepsinogen production. Moreover, the turnover of the gastric isthmus and pit regions is very fast, supporting the idea of additional multipotent stem cells around the isthmus region. In support of this hypothesis, these slowly cycling Troy$^+$ stem cells can react to 5-fluorouracil-mediated depletion of proliferative isthmus cells with increased proliferation and rapid gland repopulation. Based on this observation, Troy$^+$ corpus stem cells were termed to be reserve stem cells in the gastric corpus unit (Fig. 2.5). Unfortunately, the exact identity of the predicted isthmus stem cells is yet to be determined. Sox2-lineage tracing experiments have demonstrated the existence of multipotent stem cells that do not exhibit any chief cell characteristics [50]. If there are two or even more stem cell populations in the gastric corpus gland, it will be interesting to understand how multipotent stem cell populations located in the Isthmus and Troy$^+$ reserve stem cell populations located in the base act together to govern tissue homeostasis and injury response of the gastric epithelium.

Like the Lgr5$^+$ pyloric stem cells, single Troy$^+$ chief cells are able to give rise to gastric organoids when cultured under specific culture conditions [25]. These Troy$^+$ stem cell-derived gastric corpus organoids contain multiple other corpus epithelial

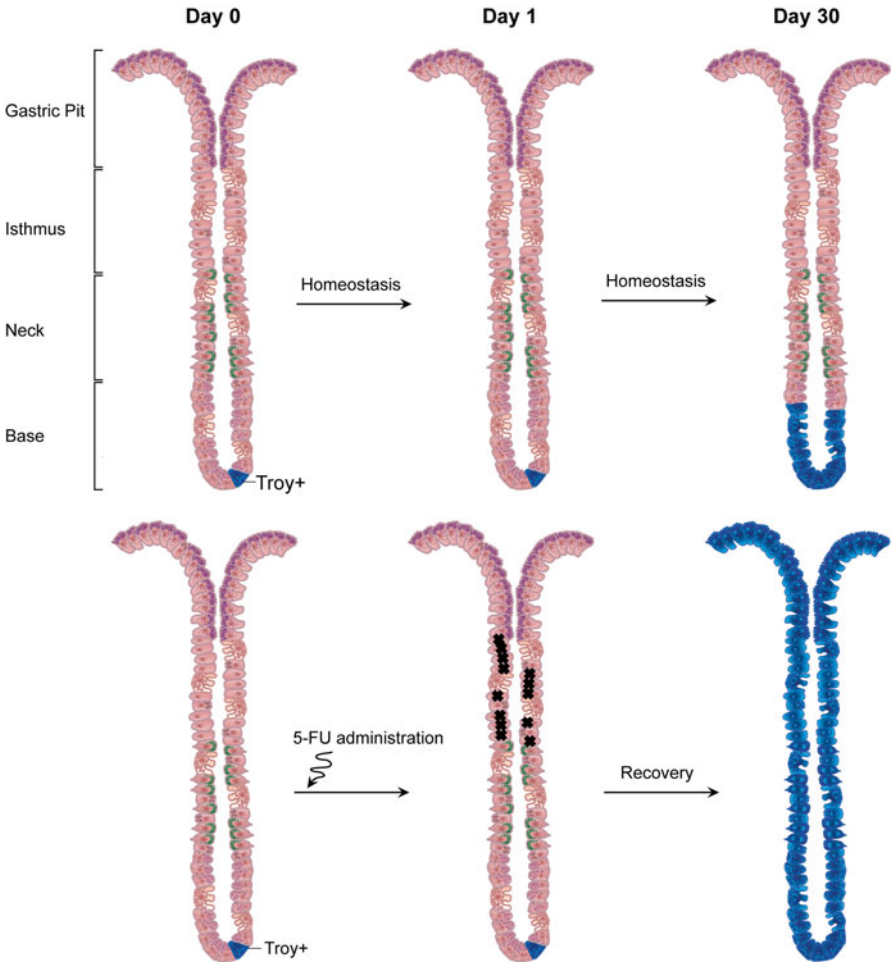

Fig. 2.5 5-FU-mediated activation of reserve stem cells in the corpus. Troy$^+$ chief cells (genetically labeled by lineage tracing at d0 in *blue*) are long-lived, fully differentiated cells located at the base of corpus glands. These cells have been shown to represent a reserve stem cell population that is mainly quiescent during homeostasis (*top panel*), but that can be reactivated if proliferative cells of the isthmus are experimentally depleted by 5-FU administration (*bottom panel*). Under these conditions Troy$^+$ reserve stem cells start cycling and generate all the cell types of the corpus gland within several weeks

cell types (mucous neck cells and pit cells) under various culture conditions, suggesting a well-retained multipotency. However, unlike their quiescent counterpart in vivo, cultured Troy$^+$ cells proliferate rapidly while maintaining Troy expression as well as chief cell characteristics. This implies that Troy$^+$ stem cells also have no intrinsic limit to their proliferation. The quiescent behavior of Troy$^+$ stem cells in vivo must be due to an unknown niche signal. By relieving this restriction, we can

now culture this interesting type of adult stem cells for many passages in vitro. Alternatively, if there is no such repressive signal in vivo, then one of the growth factors in the specific culture medium might be an activating factor for this otherwise quiescent stem cell population. Studying the exact molecular nature of the switch of this stem cell behavior (quiescence vs. active cell cycle) will help to understand the complex clone dynamics in the corpus gland of the stomach.

References

1. He XC, Zhang J, Tong W-G, Tawfik O, Ross J, Scoville DH, et al. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. Nat Genet. 2004;36:1117–21.
2. Potten CS, Hume WJ, Reid P, Cairns J. The segregation of DNA in epithelial stem cells. Cell. 1978;15:899–906.
3. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. Am J Anat. 1974;141:461–79.
4. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am J Anat. 1974;141:537–61.
5. Potten CS, Owen G, Booth D. Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci. 2002;115:2381–8.
6. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449:1003–7.
7. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature. 2011;469:415–8.
8. Cai C-L, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, et al. A myocardial lineage derives from Tbx18 epicardial cells. Nature. 2008;454:104–8.
9. Chen D, Livne-bar I, Vanderluit JL, Slack RS, Agochiya M, Bremner R. Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. Cancer Cell. 2004;5:539–51.
10. Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T, et al. Corneal avascularity is due to soluble VEGF receptor-1. Nature. 2006;443:993–7.
11. Thompson H, Tucker AS. Dual origin of the epithelium of the mammalian middle ear. Science. 2013;339:1453–6.
12. Ruppel KM, Willison D, Kataoka H, Wang A, Zheng Y-W, Cornelissen I, et al. Essential role for Galpha13 in endothelial cells during embryonic development. Proc Natl Acad Sci U S A. 2005;102:8281–6.
13. Snippert HJ, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N, et al. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science. 2010;327:1385–9.
14. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira S. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466:829–34.
15. Ahn S, Joyner AL. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. Cell. 2004;118:505–16.
16. Delacour A, Nepote V, Trumpp A, Herrera PL. Nestin expression in pancreatic exocrine cell lineages. Mech Dev. 2004;121:3–14.
17. Tang W, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, et al. White fat progenitor cells reside in the adipose vasculature. Science. 2008;322:583–6.
18. Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature. 2005;437:894–7.

19. Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun. 1997;237:752–7.
20. Barker N, van Oudenaarden A, Clevers H. Identifying the stem cell of the intestinal crypt: strategies and pitfalls. Cell Stem Cell. 2012;11:452–60.
21. De Lau W, Kujala P, Schneeberger K, Middendorp S, Li VSW, Barker N, et al. Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts". Mol Cell Biol. 2012;32:3639–47.
22. Gerbe F, van Es JH, Makrini L, Brulin B, Mellitzer G, Robine S, et al. Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. J Cell Biol. 2011;192:767–80.
23. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell. 2010;6:25–36.
24. Fafilek B, Krausova M, Vojtechova M, Pospichalova V, Tumova L, Sloncova E, et al. Troy, a tumor necrosis factor receptor family member, interacts with lgr5 to inhibit wnt signaling in intestinal stem cells. Gastroenterology. 2013;144:381–91.
25. Stange DE, Koo B-K, Huch M, Sibbel G, Basak O, Lyubimova A, et al. Differentiated troy(+) chief cells act as reserve stem cells to generate all lineages of the stomach epithelium. Cell. 2013;155:357–68.
26. Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell. 2010;143:134–44.
27. Kozar S, Morrissey E, Nicholson AM, van der Heijden M, Zecchini HI, Kemp R, et al. Continuous clonal labeling reveals small numbers of functional stem cells in intestinal crypts and adenomas. Cell Stem Cell. 2013;13:626–33.
28. Ritsma L, Ellenbroek SIJ, Zomer A, Snippert HJ, de Sauvage FJ, Simons BD, et al. Intestinal crypt homeostasis revealed at single-stem-cell level by in vivo live imaging. Nature. 2014;507:362–5.
29. Pellegrinet L, Rodilla V, Liu Z, Chen S, Koch U, Espinosa L, et al. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology. 2011;140:1230–1240.e1–7.
30. Farin HF, Van Es JH, Clevers H. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. Gastroenterology. 2012;143:1518–1529.e7.
31. Kim T-H, Escudero S, Shivdasani RA. Intact function of Lgr5 receptor-expressing intestinal stem cells in the absence of Paneth cells. Proc Natl Acad Sci U S A. 2012;109:3932–7.
32. Durand A, Donahue B, Peignon G, Letourneur F, Cagnard N, Slomianny C, et al. Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1). Proc Natl Acad Sci U S A. 2012;109:8965–70.
33. Yilmaz ÖH, Katajisto P, Lamming DW, Gültekin Y, Bauer-Rowe KE, Sengupta S, et al. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature. 2012;486:490–5.
34. Simons BD, Clevers H. Strategies for homeostatic stem cell self-renewal in adult tissues. Cell. 2011;145:851–62.
35. Simons BD, Clevers H. Stem cell self-renewal in intestinal crypt. Exp Cell Res. 2011;317:2719–24.
36. Snippert HJ, Schepers AG, van Es JH, Simons BD, Clevers H. Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. EMBO Rep. 2014;15:62–9.
37. Vermeulen L, Morrissey E, van der Heijden M, Nicholson AM, Sottoriva A, Buczacki S, et al. Defining stem cell dynamics in models of intestinal tumor initiation. Science. 2013;342:995–8.
38. Buczacki SJ, Zecchini HI, Nicholson AM, Russell R, Vermeulen L, Kemp R, Nature Publishing Group, et al. Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature. 2013;495:65–9.

39. Van Es JH, Sato T, van de Wetering M, Lyubimova A, Nee ANY, Gregorieff A, Nature Publishing Group, et al. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. Nat Cell Biol. 2012;14:1099–104.
40. Lee ER, Trasler J, Dwivedi S, Leblond CP. Division of the mouse gastric mucosa into zymogenic and mucous regions on the basis of gland features. Am J Anat. 1982;164:187–207.
41. Hattori T, Fujita S. Tritiated thymidine autoradiographic study on cellular migration in the gastric gland of the golden hamster. Cell Tissue Res. 1976;172:171–84.
42. Leblond CP, Stevens CE, Bogoroch R. Histological localization of newly-formed desoxyribonucleic acid. Science. 1948;108:531–3.
43. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. V. Behavior of entero-endocrine and caveolated cells: general conclusions on cell kinetics in the oxyntic epithelium. Anat Rec. 1993;236:333–40.
44. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. III. Inward migration of neck cells followed by progressive transformation into zymogenic cells. Anat Rec. 1993;236:297–313.
45. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. Anat Rec. 1993;236:280–96.
46. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. Anat Rec. 1993;236:259–79.
47. Lee ER, Leblond CP. Dynamic histology of the antral epithelium in the mouse stomach: II. Ultrastructure and renewal of isthmal cells. Am J Anat. 1985;172:205–24.
48. Bjerknes M, Cheng H. Multipotential stem cells in adult mouse gastric epithelium. Am J Physiol Gastrointest Liver Physiol. 2002;283:G767–77.
49. Leushacke M, Ng A, Galle J, Loeffler M, Barker N. Lgr5+ gastric stem cells divide symmetrically to effect epithelial homeostasis in the pylorus. Cell Rep. 2013;5:349–56.
50. Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, et al. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell. 2011;9:317–29.
51. Koo B-K, Clevers H. Stem cells marked by the R-spondin receptor LGR5. Gastroenterology. 2014;147:289–302.

Chapter 3
The Complex, Clonal, and Controversial Nature of Barrett's Esophagus

James A. Evans and Stuart A.C. McDonald

Introduction

Barrett's esophagus (BO) is a common preneoplastic condition, affecting approximately 1.5 million people in the UK [1]. The condition is classically described as the replacement of the stratified squamous epithelium of the distal esophagus with a distinctive columnar epithelium rich in goblet cells, so-called specialized intestinal metaplasia (IM). The presence of IM is just one possible histological finding with a diverse mixture of metaplastic glands resembling either gastric or intestinal-type enterocyte-bearing epithelium being recognized to occur in BO. It is the increased risk of malignant progression associated with the presence of goblet cells that has until recently been considered key to diagnosing BO. However, opinion on this is divided and unlike in the Unites States where goblet cells are essential for a diagnosis of BO to be made, this is not a prerequisite for BO diagnosis according to UK and Japanese guidelines [1, 2]. BO is the major precursor condition to esophageal adenocarcinoma (OAC), which is the 6th commonest cause of death in Western males [3]. OAC has a bleak 12 % survival at 5 years in the UK [1]. Although the worldwide incidence of OAC may be starting to plateau, obesity and alcohol consumption, especially in Caucasian middle-aged men, are thought to underlie an epidemic of BO in the Western hemisphere. Estimates suggest that patients with BO have a 15- to 30-fold increased relative lifetime risk of developing OAC, but only 0.12–0.3 % of BO patients per annum will actually go on to develop OAC [2, 3]. This leaves the vast majority of patients who will never develop OAC undergoing regular testing that is invasive to the patient and a financial burden on healthcare resources.

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Research in the field of Barrett's has historically focused on identifying the early cellular and genomic changes that underpin the formation of BO in the hope that this will enable better risk stratification tools for focused screening of BO patients. Typically, this involved the identification of mutations in biopsy specimens from patients undergoing surveillance for Barrett's. Historically these methods have demonstrated early inactivation of *CDKN2A* [4, 5] and further expansion of subclones with *TP53* inactivation and frequent copy number alterations and genomic doublings [6, 7]. Advances in molecular methods, particularly the development of next-generation sequencing (NGS), have permitted a deeper understanding of the epigenetic and genetic changes occurring across the genome in the evolution of BO to OA [8–10]. This review aims to examine the process by which clones of mutated stem cells expand to populate stretches of Barrett's mucosa and how this process can be studied in preneoplastic BO.

The Basic Unit of the Human GI Tract Mucosa Is the Gland

Analogous to the glands or "crypts" in the colon and small intestine, the basic stem cell unit of Barrett's epithelium is the individual gland [11]. To better understand the development of BO and its progression to OAC, researchers have sought to establish the type and location of stem cells in BO epithelium [12, 13]. While the cell of origin from which BO glands develop remains elusive, research has suggested that unlike in the colon where the stem cell marker Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is seen at the base of the crypt [14], in Barrett's glands LGR5 expression is seen about one-third up the Barrett's glandular axis at the glandular neck (or isthmus) [13]. Cellular proliferation, as demonstrated by Ki67 labeling, is also found in this region of the gland, again supporting the notion that proliferative progenitor cells reside at the Barrett's gland isthmus [13].

An in vivo cell labeling study was carried out wherein Iododeoxyuridine (IdU) was given intravenously to patients scheduled for esophagectomy. IdU (like BrDU) is incorporated into DNA during S phase of the cell cycle and thus labels dividing cells throughout the body. Patients underwent their resection anywhere between 1 and 10 weeks after IdU labeling. This made it possible to review the migration of labeled cells after IdU incorporation in the surgical resection specimen of these patients. Labeled foveolar cells migrating upward were noted to have shed to the lumen by 11 days, whereas labeled cells migrating to the gland base were much slower and still visible at 67 days postlabeling [15]. This bidirectional pattern of migration was also demonstrated in gastric corpus glands, suggesting that bidirectional cell flux is a unifying feature of Barrett's glands and gastric glands. Functionally, Barrett's glands mimic the stem cell organization of pyloric and cardia-type stomach glands. This resemblance supports the concept that Barrett's esophagus may originally be of proximal gastric epithelial origin.

Mature Barrett's glands classically contain both foveolar cells and goblet cells that descend from the luminal surface in complex, often rotated and branched

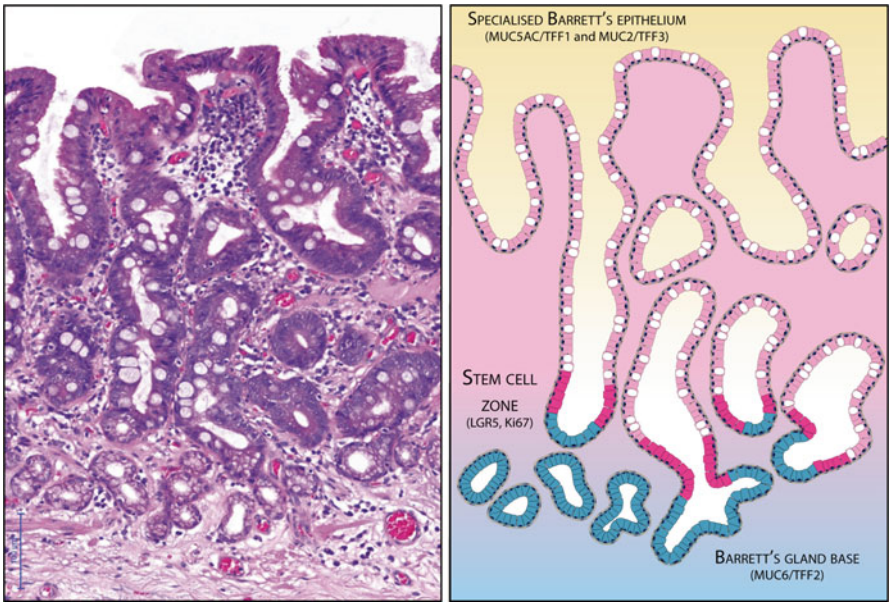

Fig. 3.1 The canonical Barrett's gland. H&E photomicrograph of nondysplastic Barrett's glands demonstrating abundant goblet cells and foveolar cells. Note the stem cell zone and mucous glands arranged as small acini at the base of these Barrett's glands. Labeling studies demonstrate bidirectional flow from the stem cell compartment. MUC5A$^+$/TFF1$^+$ foveolar cells and MUC2$^+$/TFF3$^+$ goblet cells (shown in *pink*) migrate toward the luminal surface, while MUC6$^+$/TFF2$^+$mucous cells (shown in *blue*) migrate toward the glandular base at a much slower rate. This functional compartmentalization replicates pyloric-type gastric epithelium

invaginations with mucous glands arranged as small acini at the gland bases as seen in Fig. 3.1. The mucin core proteins shown are reliable markers of intestinal and gastric differentiation [13, 16]. Both intestinal (MUC2-expressing goblet cells) and gastric differentiated cell types (MUC5AC-expressing foveolar cells at the gland surface and MUC6-expressing mucous secreting cells at the gland base) are found in specialized Barrett's glands.

Gland Phenotypes in Barrett's esophagus

Genotypic changes during BO progression have been extensively studied, but how these changes relate to changes in phenotype is unknown. BO is classically described as a "specialized" metaplasia, where metaplastic glands abundant in goblet cells resembling IM of the stomach have replaced the stratified squamous epithelium of the distal esophagus. However, this is an oversimplification, with histological material taken from BO segments exhibiting a phenotypic spectrum of at least five distinct gland phenotypes. Figure 3.2 shows the five different gland phenotypes and the

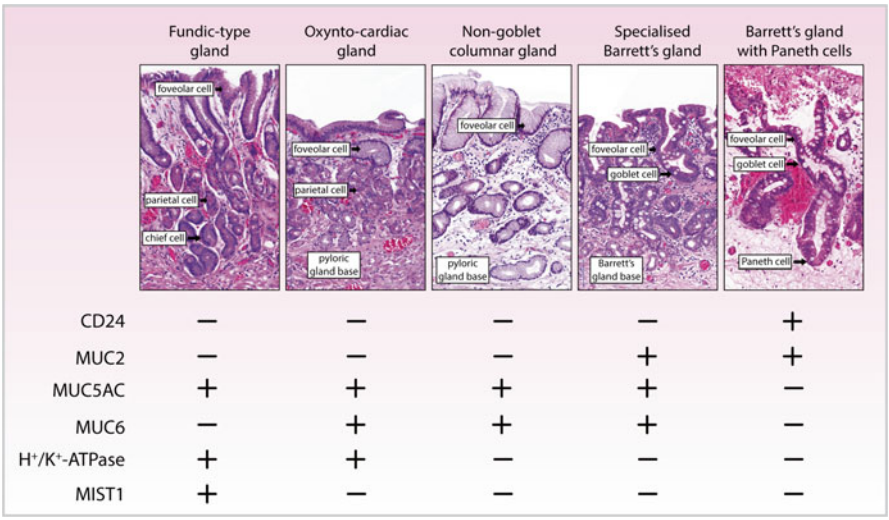

Fig. 3.2 The diversity of gland phenotypes in Barrett's esophagus. H&E photomicrographs from left to right: mature corpus glands display chief cell and parietal cell differentiation; oxyntocardiac glands (atrophic corpus glands) demonstrating parietal cells with pyloric-like gland bases, chief cells are absent; cardia-type glands are nongoblet cell-containing columnar glands with pyloric bases; specialized Barrett's glands with goblet cells and foveolar cells in the same gland; mature intestinal metaplasia complete with goblet cells and Paneth cells. Adapted from [16]. Each gland phenotype can be immunolabeled by specific cell lineage antibodies. CD24—Paneth cells, MUC2—goblet cells, MUC5AC foveolar cells, MUC6—mucous secreting cells, H^+K^+ATPase—parietal cells and MIST1—Chief cells

lineage differentiation markers that identify them. These phenotypes encompass glands that contain mature gastric body-type differentiated cells (such as acid-secreting parietal cells and pepsin-secreting chief cells) through to mature intestinal glands that exhibit Paneth cells. Biopsies from "Barrett's segments" may also show mature (i.e., non-atrophic) corpus-type mucosa; however, in practice, these biopsies almost invariably derive from the hiatus hernia.

The distribution of these 5 different phenotypes has been studied in patients. A random distribution of gland phenotypes with a lack of zonation has been reported in the literature [17, 18]. In contrast to this random mosaic of gland phenotypes, Going et al. [19] revealed a proximo-distal gradient of goblet cells along the segment with significantly greater numbers of goblet cells found in the proximal Barrett's segment, cardiac-type mucosa being found throughout the segment, and oxyntocardiac-type glands being found more distally. There are a range of possible relationships between phenotype and clonal expansion in BO. Phenotype may remain fixed and independent of clonal evolution, or demonstrate a plasticity of phenotype where environmental signals alter phenotype over time as the genotype evolves. There is evidence of how these phenotypes develop over time with a sequence of phenotypic evolution being hypothesized. Following esophagectomy where the proximal stomach is anastomosed to the remaining proximal esophagus,

columnar mucosa develops in the squamous-covered esophageal remnant. The first phenotype to develop within 1–2 years is a cardiac type where simple mucinous glands lacking parietal cells are seen. After 3–5 years postsurgery phenotypic evolution to goblet cell differentiation occurs with expression of CDX2 and MUC2. It has been demonstrated that CDX2 and Villin expression have a key role in signaling intestinal differentiation and subsequent goblet cell metaplasia in BO. Hahn et al. studied 89 BO cases of which 59 cases contained goblet cells. Ninety-eight percent of cases with goblet cells showed CDX2 expression, whereas only 43 % of patients without goblet cells expressed CDX2 [16]. Villin expression occurred in 17 % of these nongoblet cell cases. This study however suggests that in spite of the absence of goblet cell differentiation metaplastic esophageal columnar epithelium can show "fruste" intestinal differentiation [16].

To summarize, a phenotypic sequence can be proposed with initial nongoblet columnar-lined mucosa, leading to glands acquiring intestinal gene expression and finally to "specialized" metaplastic glands. This phenotype may extend proximally in response to bile salt-induced CDX2 expression. Although clear anatomical and mechanistic differences exist, evidence of a proximally migrating columnar epithelium has been provided in a study utilizing transgenic $p63^{null}$ mice. Investigators demonstrated the progressive replacement of eroded squamous epithelium with proximal shift in the level of the squamocolumnar junction by stomach-derived columnar epithelium [20]. Thus, it is suggested that the effects of acid reflux erode the squamous epithelium allowing proximal expansion of columnar epithelium. This proximal expansion may facilitate clonal expansion of preexistent oncogenic mutations.

However, regardless of the definitive clonal origin of Barrett's epithelium, this phenotypic spectrum of glands in BO as it appears in clinical specimens is rarely appreciated. We therefore submit that there is a paucity of research coupling phenotypic to genotypic changes during BO progression.

The Stem Cell Niche and Niche succession in Barrett's

The stem cells in BO, like other GI epithelia, exist in a conceptual physical space often described as the stem cell "niche." The niche encapsulates all resident stem cells and the mesenchymal cells that surround the dedicated epithelial stem cells and it cooperatively regulates how stem cell proliferation takes part in tissue generation, maintenance, and repair. Consequently, the niche has both functional and anatomical dimensions [21]. Stem cells can divide either asymmetrically producing one daughter and one stem cell, or symmetrically producing either two daughter cells or two stem cells. These two types of stem cell divisions will eventually result in one stem cell and its progeny becoming dominant in the stem cell niche in a process known as "niche succession" [22]. Applying this concept to Barrett's glands, through a process of niche succession the progeny of a single stem cell will come to populate an entire Barrett's gland. This process is termed monoclonal conversion as

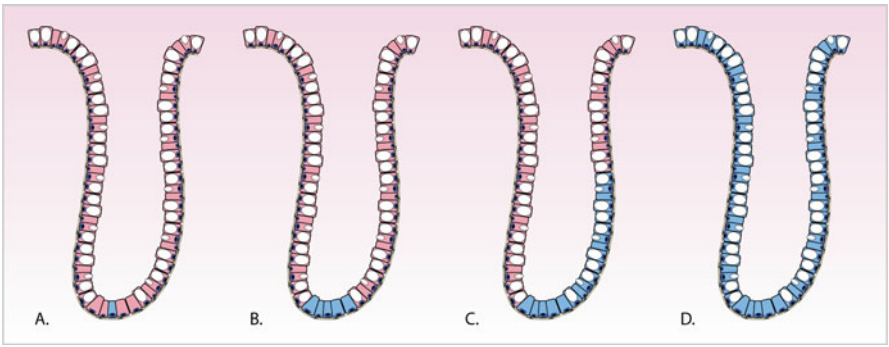

Fig. 3.3 Monoclonal coversion. (**a**) New stem cell lineage arising in the gland base. (**b**) Through neutral drift this clone expands to occupy the gland base, (**c**) via niche succession further expansion occurs until (**d**) all cells in the gland share the same mutation. This is when monoclonal conversion has occurred

every cell in the gland has arisen from the same original stem cell, with the gland becoming a single clonally derived unit [23] (Fig. 3.3).

Clonal labeling methods used to identify colonic crypt stem cells have shown that only few functioning stem cells actively cycle at the crypt base [24, 25]. The term "neutral drift" is used to describe the dynamic of stem cell loss and subsequent replacement. It allows small populations of neighboring stem cells to compete to allow progenitor cells (clones) to expand or contract at random, either becoming established and surviving through monoclonal conversion or undergoing clonal extinction. Mutations that give a survival or growth advantage alter the niche succession rate and this effect is termed "biased drift." The genetic mutations that provoke biased drift are often described as "driver mutations," whereas neutral mutations have been described as "passenger mutations." Understanding how driver and passenger mutations affect the stem cell dynamics in BO first requires a better grasp of the stem cells that give rise to the lesion itself. To answer this question we must first ask: what is the clonal architecture of the Barrett's gland?; how do clones in Barrett's glands arise and expand and what methods do we have to measure this process?; finally, what dynamics underpin the fixation and competition between clones within a Barrett's gland?

Tracing Clonal Lineages in Barrett's

Attempts to study clonal conversion in normal human epithelial tissues have utilized a range of methodologies for lineage tracing, including inactivation of X-linked genes [26], microsatellite markers in UC patients who progress to cancer [27], DNA methylation signatures [28, 29], mitochondrial DNA (mtDNA) mutations, and most recently whole genome sequencing [9]. The dynamics and differentiation of somatic

stem cells in BO has been studied by exploiting the inheritance of nonpathogenic mutations in mtDNA [30]. Each cell contains multiple mitochondria with each mitochondrion containing numerous copies of its genome. Oxidative phosphorylation and a functioning electron transport chain require mitochondrial respiratory chain proteins that are both nuclear and mtDNA encoded. The mitochondrial genome contributes to all protein complexes in the mitochondrial respiratory chain, except for Complex II, which is entirely encoded by the nuclear genome. Tumor-associated genomic mutations may influence clonal expansion rates, but mtDNA mutations are considered useful lineage markers as they are felt to be neutral in their effect on clonal expansion. Spontaneously occurring mutations in mtDNA can slowly expand within stem cell populations allowing the dynamic behavior of long-lived stem cells within the niche to be traced. Through mitochondrial duplication and attrition mutations may expand to a state of "fixation," whereby either some mitochondria (heteroplasmy) or potentially all of the mitochondria within a cell (homoplasmy) contain the same mutation.

The mitochondrially encoded Cytochrome c Oxidase gene (CCO) forms the last stage of the electron transport chain in respiratory Complex IV. When over 80 % of the mitochondrial genomes in a cell contain the same CCO mutation the enzymatic function of CCO is abolished. Identification of CCO-deficient clones or groups of CCO-deficient cells is achieved through the use of a dual color enzyme histochemistry staining. The dual color staining protocol facilitates brown and blue staining in a tissue section, where brown staining indicates CCO substrate conversion and retention of Complex IV function—the absence of brown staining (i.e., loss of CCO substrate conversion due to loss of Complex IV enzymatic function) is highlighted by the blue counterstain. This blue counterstain reveals succinate dehydrogenase (Complex II) activity. Together the blue staining thus demonstrates a loss of function of the mitochondrial enzymes of the chain, because the succinate dehydrogenase complex II activity is fully nuclear encoded. These blue CCO-deficient groups of cells (or patches) can be laser capture microdissected and subjected to mtDNA sequencing. If the same mutation is: (1) present in all CCO-deficient cells in a patch and; (2) absent in all brown (CCO-proficient) patches then the epithelial cell population is clonal in origin.

Studies of the clonal architecture of the human stomach utilizing CCO mutations as clonal markers have shown that the gastric gland behaves as a clonal unit. In the stomach the gland neck and foveolus were defined as the gastric unit analogous to the entire crypt in the human colon. The same CCO mutation was found throughout an entirely CCO-deficient gastric unit [31]. Thus, all differentiated progeny in the gastric unit were shown to arise from the same stem cell. Furthermore, intestinal metaplasia (IM) glands from patients undergoing resection for gastric adenocarcinoma were entirely CCO deficient, supporting the concept that human gastric epithelial units undergoing intestinal metaplasia are clonal units. Partially CCO-deficient glands were identified as mixed brown and blue glands thus demonstrating that multiple (or, at least two) stem cell populations are present in the niche. In the same study wholly CCO-deficient Barrett's metaplasia glands were shown to contain all the differentiated cell lineages, including goblet, foveolar, and neuroendocrine cells, supporting multilineage differentiation arising from a clonally derived population

of Barrett's gland stem cells. Further use of CCO lineage labeling by Lavery et al. [13] utilizing intestinal and gastric lineage markers (including mucin core proteins and trefoil factor peptides) combined with CCO lineage labeling demonstrated that different lineages throughout the entire length of Barrett's glands are clonally related as they all harbored the same mitochondrial mutation. Collectively, this demonstrates that BO glands contain multiple stem cells and share a common progenitor cell that is capable of giving rise to all differentiated cell types within the Barrett's gland [32].

Clonal Expansions in Non-dysplastic Barrett's Esophagus

In the context of reduced lower esophageal tone, often related to hiatus hernia formation, the mucosal lining of the distal esophagus is chronically exposed to bile and acid reflux. It is intuitive that this harsh acidic environment provides a selective advantage to a protective mucin-producing columnar epithelial cell type over the native stratified squamous epithelium. So how do we explain (at a clonal level) what has occurred when endoscopically, as is seen in some cases, very long 10–14 cm segments of columnar lined mucosa are discovered? As discussed earlier, through a process of survival of the fittest, a clear fitness advantage inferred by the columnar cell type over the squamous cell type has allowed a clone (or multiple distinct clones) of columnar cells to expand over the length of the Barrett's segment with the squamous epithelial cell population being driven to extinction.

But how does this physical expansion occur? If we look back to consider how the GI tract develops, the crypts of the colon, glands in the stomach, and the glands of the metaplastic esophagus are established via the same process of crypt fission [31–33]. This process allows division (or bifurcation) of a gland initiated at the gland base, which then separates lengthways giving rise to two separate glands (Fig. 3.4).

These basal gland divisions lead to lateral expansion via fission, but this occurs very slowly. Rates in BO are still unknown, but the average colonic crypt cycle is 36 years equating to only one or two crypt fission events per lifetime [24]. The highly complex glandular structure of BO glands has so far hindered clonal labeling studies. Unpublished 3D modeling studies of our group utilizing computer-rendered 3D imaging demonstrate a highly complex structure unique to the GI tract. The crypt-like branching bases lead up to the gland neck, often surrounded by other rotating glands that extend up to a superficial compartment opening to the lumen (our unpublished data).

Fission has been demonstrated in the stomach [31], whereby gastritis-induced IM can lead to dysplastic changes and eventually lead to gastric adenocarcinoma. Although thought to occur at a very slow rate in the normal epithelial setting, the driving effects of chronic inflammation such as that seen in ulcerative colitis or BO may speed up fission events. In the context of colonic inflammation, upregulation of the rate of crypt fission in ulcerated epithelium of patients with UC has been shown to increase over 40-fold [34]. So reflux in the context of BO is a potential means by

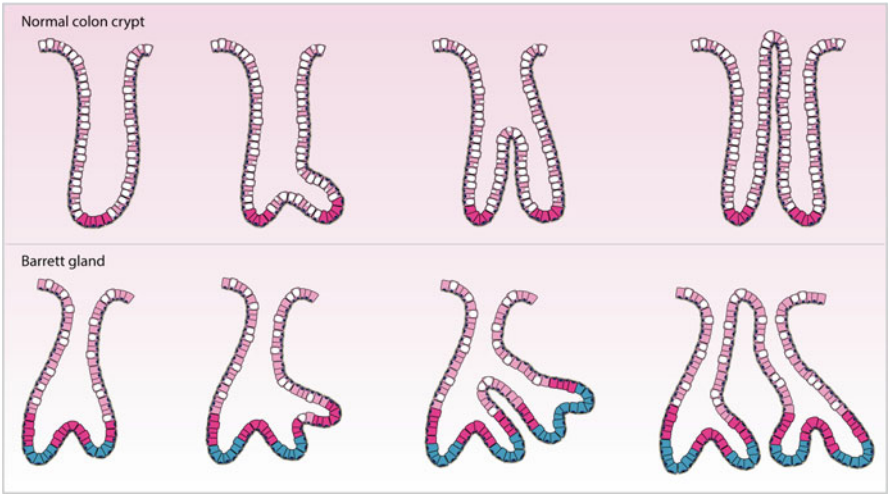

Fig. 3.4 Gland fission in GI epithelium. Stem cells in the colonic crypt base (*top*) divide with bifurcation of the crypt base and longitudinal separating upward resulting in two crypts in a process of crypt fission. Analogous bifurcation is seen in the Barrett's gland (*bottom*)

which gland fission may drive the establishment of clonal segments of Barrett's epithelium.

Although the expansion rate at which Barrett's segments are established has not been demonstrated there is some recent data on segment length. In one study, 763 patients were followed over 20 years with no significant change in length of Barrett's segment seen [35]. In a larger and more recent study, 3635 patients were followed with no significant change in segment length regardless of age [36]. These studies confirm that segment length is established by the time of initial endoscopic assessment and remains constant from thereon. The establishment of a new segment of columnar-lined esophagus in response to gastric reflux-driven inflammation may provide the opportunity for the establishment of a clonal field of which a subset of clones in a small proportion of patients may go on to evolve to OAC. Interactions between competing clones may be the significant factor driving cancer formation via clonal competition, cooperation, or other interactions between clonal populations.

Clonal Dynamics and Expansion in Barrett's Esophagus

First suggested by Nowell in 1975, mutations accumulating in a single tissue stem cell may infer a fitness advantage to promote enhanced growth and survival that is passed on to all progeny of this cell allowing a mutant clone to be established within a tissue [37]. This led to the hypothesis that the majority of cells within a Barrett's segment were derived from a single founder cell. Indeed, early studies reinforced

this hypothesis; Barrett et al. used LOH analysis to show that genetic variation occurred after a founder clone had expanded throughout the entire lesion [38]. Further studies were also able to demonstrate this by showing that purified biopsy specimens taken at different levels within the esophagus were clonal [5, 6]. It was therefore thought that BO progressed to cancer through a number of clonal selective sweeps, where mutations spread throughout a segment to "fixation" with all glands containing the same mutation. Against this theory are the findings by Leedham et al. who sampled resection specimens and individual glands from biopsies rather than purified biopsy specimens [39]. This study demonstrated that rather than a single founder mutation sweeping through an entire Barrett's segment, marked clonal heterogeneity with multiple independent clones exist in BO. Newer high resolution whole genome sequencing utilizing NGS methods have further confirmed this heterogenous landscape in nondysplastic BO. Utilizing paired samples of BO and OAC samples from 23 patients multiple clones were shown to be present in nondysplastic biopsy samples [8].

If clonal expansion underlies the early events in driving nondysplastic Barrett's to cancer then the promotion of which clone (or clones) rises to the top of the evolutionary fitness peak is certainly impacted upon by the diversity between clones within the Barrett's segment. Even when taking into account known genetic risk factors, including lesions in *TP53* and copy number abnormalities, diversity has been shown to be a strong predictor of risk of progression in BO [40]. Maley et al. utilized diversity measures from studies in evolutionary biology to measure the number of clones present in biopsies from 268 affected individuals over at least two time points [40]. These measures of clonal diversity could even predict which patients were most likely to progress to OAC.

Li et al. prospectively compared somatic chromosomal alterations in 169 "non-progressors" and 79 "progressors" who went on to develop OAC. Genomes of non-progressors remained relatively stable. However in progressors, sudden punctuations and large clonal expansions involving catastrophic genomic doubling were found to be occurring in a relatively short window only 2–4 years prior to OAC development [7]. One note of caution when interpreting this data is the high progression rate of 37/268 cases over 4.4 year time period, which is very much higher than current estimated progression rates (<0.2 %/year). With most progressors in this study being recruited less than 48 months before the development of OAC from endoscopy performed between 1988 and 2009, it cannot be excluded that some of these cases reflect missed "interval" cancers where the first endoscopy was falsely negative. Significant developments in optical enhancement and chromoendoscopy developed in the last 5 years allowing previously missed flat dysplasia to be detected will certainly ensure that neoplastic clones are detected at earlier stages. In future studies this will further reduce the progression rate. These considerations notwithstanding, it is suggested that a situation of relative stasis and equilibrium exists with abrupt chromosomal alterations occurring in those patients who progress to OAC. Dysplastic precancer clones may not necessarily confer a competitive advantage. Longitudinal

studies in the colon have shown that many neoplastic precursor lesions (tubular adenomas) will regress at some point after initiation [33, 41], indicating that the ratio of dysplastic precursor clones to clinically relevant OAC may be greater than 1:1. We would need similar longitudinal follow-up studies in Barrett's patients to trace the dynamic behavior of dysplastic clones. It is not inconceivable that many of these clones are driven to extinction before one clone "successfully" progresses to OAC. This could provide important new insights on the development of chemopreventive strategies.

Combining Clonal Labeling with Phenotype

Employing the mtDNA CCO mutational labeling approach to trace clonal ancestry combined with somatic point mutation analysis, Lavery et al. have recently demonstrated that clonal expansion and progression to cancer in BO is not exclusive to intestinal metaplasia [42]. Utilizing a longitudinally frozen esophagectomy specimen spanning the squamocolumnar junction to the stomach, a focus of OAC was shown to have evolved from nondysplastic cardia-type columnar mucosa with the same CCO mutation and somatic *TP53* mutations found in the OAC and nongoblet cell epithelium and distant liver metastases. These mutations were not found in the goblet cell epithelium in this Barrett's segment (Fig. 3.5). This data is the first demonstration that expansion of nongoblet cell epithelium can give rise to OAC.

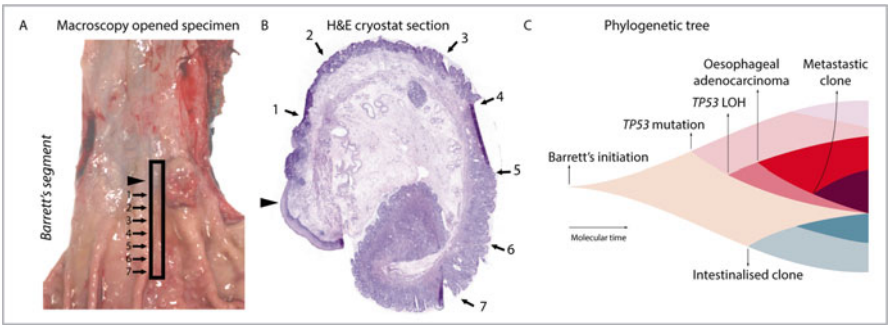

Fig. 3.5 Spatial sampling of a Barrett's segment and associated esophageal adenocarcinoma (OAC). Adapted from [40] (**a**) Longitudinal opened resection specimen (rectangle) with columnar metaplasia across the gastro-esophageal junction (*arrows*) and nodular OAC (*arrowhead*). (**b**) H&E-stained cryostat section (*left*) of the longitudinal strip across the gastro-esophageal junction reveals columnar metaplasia of the distal esophagus (*arrows*) and an OAC at the squamocolumnar junction (*arrowhead*). Submucosal gland complex (*asterisk*) confirms the esophageal origin. (**c**) Phylogenetic tree of the clonal evolution of this OAC from metaplastic columnar epithelium without goblet cells in BO. From a common progenitor two metaplastic clones are detected within this Barrett's segment. Expansion of clones is associated with further subclonal evolution. One subclone within the metaplastic columnar epithelium without goblet cells acquired a *TP53* mutation and eventually gave rise to metastatic esophageal adenocarcinoma

Clonal Expansion Postradiofrequency Ablation (RFA)

Another factor governing the rate of clonal expansion in BO are the iatrogenic effects of endoscopic mucosal resection (EMR) and radiofrequency ablation (RFA Patients who progress to nodular Barrett's dysplasia are typically offered EMR followed by RFA of the entire Barrett's lesion [1]. The ablation of columnar mucosa is designed to allow reepithelialization with squamous mucosa. While these procedures are effective in eliminating Barrett's related dysplasia, there is a demonstrable failure rate with recurrence of Barrett's metaplasia in up to 20–45 % over time. Zeki et al. showed that 5 of 19 patients who had undergone EMR followed by RFA therapy were found to harbor de novo mutations in nondysplastic Barrett's epithelium and greater clonal diversity [43]. The use of RFA may thus initiate a potential genetic bottleneck and provoke dysplasia progression in a clone independent from the original dysplastic clone.

Conclusion

In summary, measuring clonal dynamics in Barrett's remains more elusive than the studies of native intestinal stem cell dynamics in, for example, the colon due to the complexity of the Barrett's gland itself and the range of phenotypes that exist. Further research aims to tackle these issues and improve the detection and subsequent treatment of OAC and its precursor lesion BO. What has now been conclusively demonstrated is that Barrett's glands, like other glandular units in the GI tract are clonally derived units. In Barrett's glands a single stem cell lineage can give rise to all the differentiated epithelial cell types seen within the stereotypical Barrett's gland. Lineage tracing analysis has revealed that Barrett's glands are capable of bifurcation and that BO in some patients is a complex, oligoclonal lesion. BO is phenotypically diverse with a range of glandular phenotypes. Recent work now shows the development of OAC from a nongoblet cell epithelium with potential implications for diagnostic and surveillance policy. Life-long follow-up of patients who have undergone RFA is also important due to the potential recurrence of dysplasia from novel clones attributed to the clonal bottleneck effect induced by the ablation of the Barrett's field.

References

1. Fitzgerald RC, et al. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. Gut. 2014;63(1):7–42.
2. Zeki S, Fitzgerald RC. Targeting care in Barrett's oesophagus. Clin Med. 2014;14 Suppl 6:s78–83.
3. Hvid-Jensen F, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011;365(15):1375–83.

4. Hardie LJ, et al. p16 expression in Barrett's esophagus and esophageal adenocarcinoma: association with genetic and epigenetic alterations. Cancer Lett. 2005;217(2):221–30.
5. Wong DJ, et al. p16(INK4a) lesions are common, early abnormalities that undergo clonal expansion in Barrett's metaplastic epithelium. Cancer Res. 2001;61(22):8284–9.
6. Galipeau PC, et al. Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl Cancer Inst. 1999;91(24):2087–95.
7. Li X, et al. Temporal and spatial evolution of somatic chromosomal alterations: a case-cohort study of Barrett's esophagus. Cancer Prev Res (Phila). 2014;7(1):114–27.
8. Ross-Innes CS, et al. Whole-genome sequencing provides new insights into the clonal architecture of Barrett's esophagus and esophageal adenocarcinoma. Nat Genet. 2015;47(9): 1038–46.
9. Weaver JM, et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet. 2014;46(8):837–43.
10. Stachler MD, et al. Paired exome analysis of Barrett's esophagus and adenocarcinoma. Nat Genet. 2015;47(9):1047–55.
11. McDonald SA, et al. Barrett oesophagus: lessons on its origins from the lesion itself. Nat Rev Gastroenterol Hepatol. 2015;12(1):50–60.
12. Barbera M, et al. The human squamous oesophagus has widespread capacity for clonal expansion from cells at diverse stages of differentiation. Gut. 2015;64(1):11–9.
13. Lavery DL, et al. The stem cell organisation, and the proliferative and gene expression profile of Barrett's epithelium, replicates pyloric-type gastric glands. Gut. 2014;63(12):1854–63.
14. Baker AM, et al. Characterization of LGR5 stem cells in colorectal adenomas and carcinomas. Sci Rep. 2015;5:8654.
15. Pan Q, et al. Identification of lineage-uncommitted, long-lived, label-retaining cells in healthy human esophagus and stomach, and in metaplastic esophagus. Gastroenterology. 2013;144(4): 761–70.
16. Hahn HP, et al. Intestinal differentiation in metaplastic, nongoblet columnar epithelium in the esophagus. Am J Surg Pathol. 2009;33(7):1006–15.
17. Gottfried MR, McClave SA, Boyce HW. Incomplete intestinal metaplasia in the diagnosis of columnar lined esophagus (Barrett's esophagus). Am J Clin Pathol. 1989;92(6):741–6.
18. Thompson JJ, Zinsser KR, Enterline HT. Barrett's metaplasia and adenocarcinoma of the esophagus and gastroesophageal junction. Hum Pathol. 1983;14(1):42–61.
19. Going JJ, et al. Zoning of mucosal phenotype, dysplasia, and telomerase activity measured by telomerase repeat assay protocol in Barrett's esophagus. Neoplasia. 2004;6(1):85–92.
20. Wang X, et al. Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell. 2011;145(7):1023–35.
21. Yen TH, Wright NA. The gastrointestinal tract stem cell niche. Stem Cell Rev. 2006;2(3):203–12.
22. Leedham SJ, et al. Gastrointestinal stem cells and cancer: bridging the molecular gap. Stem Cell Rev. 2005;1(3):233–41.
23. Zeki SS, Graham TA, Wright NA. Stem cells and their implications for colorectal cancer. Nat Rev Gastroenterol Hepatol. 2011;8(2):90–100.
24. Kozar S, et al. Continuous clonal labeling reveals small numbers of functional stem cells in intestinal crypts and adenomas. Cell Stem Cell. 2013;13(5):626–33.
25. Lopez-Garcia C, et al. Intestinal stem cell replacement follows a pattern of neutral drift. Science. 2010;330(6005):822–5.
26. Novelli M, et al. X-inactivation patch size in human female tissue confounds the assessment of tumor clonality. Proc Natl Acad Sci U S A. 2003;100(6):3311–4.
27. Salk JJ, et al. Clonal expansions in ulcerative colitis identify patients with neoplasia. Proc Natl Acad Sci U S A. 2009;106(49):20871–6.
28. Maegawa S, et al. Widespread and tissue specific age-related DNA methylation changes in mice. Genome Res. 2010;20(3):332–40.

29. Humphries A, et al. Lineage tracing reveals multipotent stem cells maintain human adenomas and the pattern of clonal expansion in tumor evolution. Proc Natl Acad Sci U S A. 2013;110(27):E2490–9.
30. Taylor RW, et al. Mitochondrial DNA mutations in human colonic crypt stem cells. J Clin Invest. 2003;112(9):1351–60.
31. McDonald SA, et al. Mechanisms of field cancerization in the human stomach: the expansion and spread of mutated gastric stem cells. Gastroenterology. 2008;134(2):500–10.
32. Nicholson AM, et al. Barrett's metaplasia glands are clonal, contain multiple stem cells and share a common squamous progenitor. Gut. 2012;61(10):1380–9.
33. Baker AM, et al. Quantification of crypt and stem cell evolution in the normal and neoplastic human colon. Cell Rep. 2014;8(4):940–7.
34. Cheng H, et al. Crypt production in normal and diseased human colonic epithelium. Anat Rec. 1986;216(1):44–8.
35. Gatenby PA, et al. Does the length of the columnar-lined esophagus change with time? Dis Esophagus. 2007;20(6):497–503.
36. Moawad FJ, et al. Barrett's oesophagus length is established at the time of initial endoscopy and does not change over time: results from a large multicentre cohort. Gut. 2015;64(12): 1874–80.
37. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194(4260):23–8.
38. Barrett MT, et al. Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet. 1999;22(1):106–9.
39. Leedham SJ, et al. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. Gut. 2008;57(8):1041–8.
40. Maley CC, et al. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. Nat Genet. 2006;38(4):468–73.
41. Jones S, et al. Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A. 2008;105(11):4283–8.
42. Lavery DL. et al. Evolution of oesophageal adenocarcinoma from metaplastic columnar epithelium without goblet cells in Barrett's oesophagus. Gut. 2016;65:907–13.
43. Zeki SS, et al. Clonal selection and persistence in dysplastic Barrett's esophagus and intramucosal cancers after failed radiofrequency ablation. Am J Gastroenterol. 2013;108(10): 1584–92.

Chapter 4
A New Pathologic Assessment of Gastroesophageal Reflux Disease: The Squamo-Oxyntic Gap

Parakrama Chandrasoma and Tom DeMeester

Introduction

Gastroesophageal reflux disease (GORD) is regarded as a progressive disease. When defined by the presence of symptoms, most people in the population never develop GORD. Twenty to forty percent of the population develops symptomatic GORD. Approximately 70 % of these patients are well controlled throughout life with empiric treatment with proton pump inhibitors (PPI). Their disease does not seem to be progressive although some dose escalation is frequently needed for control.

From this perspective, progression is limited to the approximately 30 % of GORD patients in whom PPI therapy fails to control symptoms (Fig. 4.1). There is no ability or attempt to prevent the entry of GORD patients into this stage of treatment failure. Patients who are not well controlled with PPI live a life whose quality is compromised to varying degrees by their symptoms. It is only when they reach this stage defined by failure of PPI to control symptoms or develop alarm symptoms such as dysphagia that endoscopy is indicated [1].

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From the perspective of endoscopy, GORD progresses from no recognized endoscopic change to erosive esophagitis of increasing severity (Los Angeles grade A to D), to visible columnar lined esophagus (vCLO) and adenocarcinoma.

Biopsy is not recommended in patients who do not have an endoscopic abnormality [1]. Biopsy of the normal squamous epithelium may show histologic changes such as intraepithelial eosinophils and basal cell hyperplasia, but these are not sufficiently sensitive or specific to have practical value. Biopsy of the normal squamocolumnar junction is not recommended, although it is known that a small but significant number of patients will have intestinal metaplasia if biopsies are taken [2].

Endoscopy in the patient who has failed PPI therapy has practical value only in the detection of Barrett esophagus (Fig. 4.1). In patients without Barrett esophagus, endoscopy provides little if any useful information that improves symptom control with PPI. The detection of Barrett esophagus has value only to place patients on a surveillance protocol to detect early neoplastic change. Barrett esophagus has no effective medical treatment. Progression to dysplasia and adenocarcinoma cannot be effectively prevented [3].

Symptoms and endoscopic findings of GORD are not concordant. A person without symptoms of GORD can have long segment Barrett esophagus or present with the clinical expression of an advanced GORD-induced adenocarcinoma. Conversely, a patient with symptoms can be endoscopically normal (nonerosive reflux disease or NERD). Treatment of GORD with PPI can heal erosive esophagitis without completely resolving GORD symptoms [1]. Patients with NERD are commonly more resistant to symptom control with PPI than those with erosive esophagitis [1].

There is no symptom complex that can accurately predict who will progress to failure of medical therapy in the future. As a result, all patients receive empiric acid suppressive treatment with the sole objective of symptom control. Failure is recognized only when maximum PPI therapy fails to control symptoms. There is no symptom complex or endoscopic finding short of Barrett esophagus that can predict adenocarcinoma in the future. Screening for Barrett esophagus is not recommended [3].

This treatment algorithm therefore precludes any method that can prevent the progression of GORD to its severe end points. When the end point of severe GORD is compromised quality of life, antireflux surgery offers the only hope of control. However, surgery has its own problems and is relatively rarely performed. Many patients continue to live a life that is compromised by fear of eating, sleep deprivation, and loss of productivity at work. When the end point of severe GORD is advanced adenocarcinoma, hope exists for very few people and too commonly for a very short period of time (Fig. 4.1).

This is a sad commentary of our present management of GORD. We have abandoned the hallowed principles of early diagnosis in favor of an illogical and unrealized hope that PPI will cure the disease. We simply permit the development of severe GORD and then struggle with few good answers to impaired quality of life and progression to adenocarcinoma.

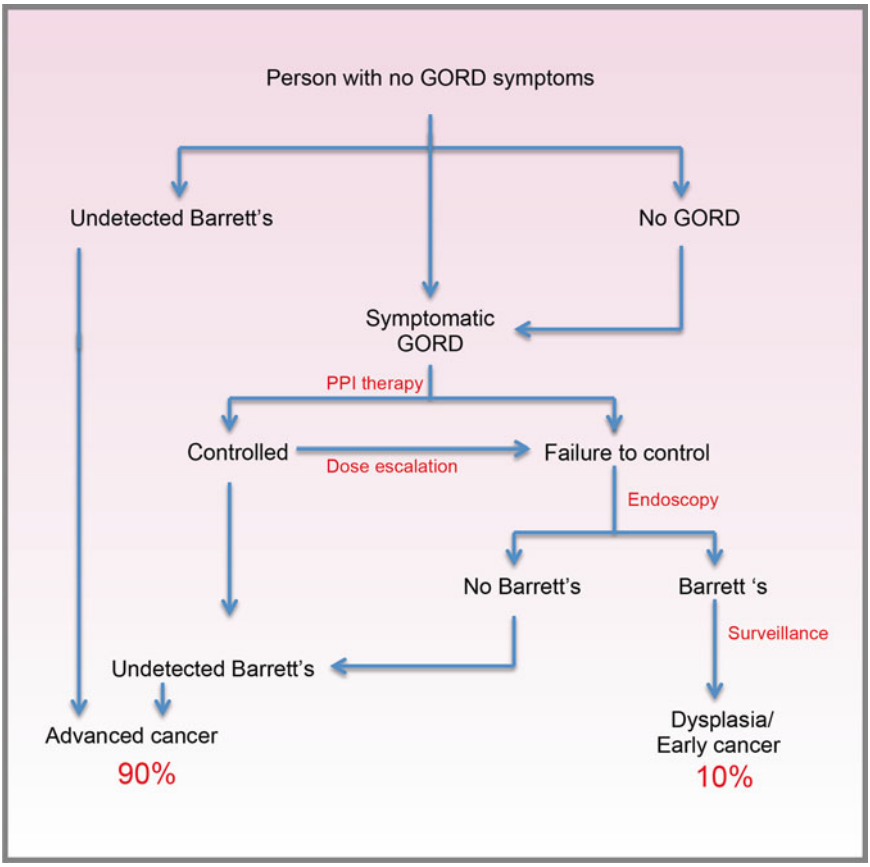

Fig. 4.1 The failure of the present treatment algorithm of GORD to prevent mortality from esophageal adenocarcinoma. Endoscopy is limited to patients who fail medical therapy and surveillance is limited to those patients who have Barrett esophagus at endoscopy. Ninety percent of adenocarcinomas occur in asymptomatic people, patients well controlled by PPI, and people that do not have Barrett esophagus at endoscopy. Only 10 % are found in early stages of cancer and can be treated effectively with a mortality of <30 % compared to 90 % for advanced cancer

Progression of GORD with Empiric PPI Therapy

The best available scientific prospective study of long-term outcomes associated with treating symptomatic GORD with acid suppressive medical therapy is the ProGERD study [4]. A total of 6215 patients over 18 years old with the primary symptom of heartburn were enrolled into this prospective multicenter open cohort study in Europe. The study was largely conducted under the auspices of Astra-Zeneca, which makes any result that suggests a negative effect of PPI therapy highly credible. All patients underwent an index endoscopy done in selected centers by endoscopists who received special training. Endoscopic findings were recorded and the

patients given 4–8 weeks of PPI therapy with assessment of symptoms control and repeat endoscopy to assess healing. They were then sent back to their primary care physicians for continuation of empiric acid suppressive treatment at their discretion. Treatment used during follow up and symptom control were monitored by questionnaires. A total of 2721 of this cohort of patients reported to the study centers for repeat endoscopic assessment at 5 years.

At the initial endoscopy, the distribution of endoscopic changes of these 2721 patients was as follows: nonerosive disease, 1224; erosive disease LA A/B, 1044; erosive disease LA C/D, 213; 240 (8.8 %) patients had vCLO (note: vCLO was reported as "Barrett esophagus, endoscopic" and "Barrett esophagus with histologic confirmation"). The patients with vCLO at the initial endoscopy were not included in this study.

Reversal and prevention of progression of erosive esophagitis at 5 years was impressive. Of the 1041 patients with nonerosive disease at baseline, 784 remained nonerosive, 248 progressed to LA A/B, and nine to LA C/D erosive disease. Of the 918 patients with LA A/B erosive disease at baseline, 578 had reversed to nonerosive disease, 331 remained LA A/B, and nine had progressed to LA C/D erosive disease. Of the 188 patients with LA C/D erosive disease at baseline, 94 now had nonerosive disease, 78 had LA A/B, and 16 stayed at LA C/D erosive disease. Over a period of 5 years, the number of patients with severe erosive esophagitis had decreased from 188 to 34. Regular intake of PPI reduced the likelihood of progression compared with on demand PPI or other therapy. The severity of symptoms at baseline was not a predictor of progression to severe erosive esophagitis. It could reasonably be concluded that PPI therapy was highly effective in healing erosive esophagitis.

In contrast, 241 (9.7 %) patients who did not have vCLO initially had developed this at 5 years. These patients who progressed included 72/1224 (5.9 %) who originally had NERD, 127/1044 (12.1 %) with LA grade A/B, and 42/213 (19.7 %) with LA grade C/D erosive esophagitis. The factors significantly associated with progression to vCLO at 5 years were as follows: (a) female gender, which had a negative association ($p=0.041$); (b) alcohol intake ($p=0.033$); (c) erosive esophagitis compared with NERD ($p<0.001$); (d) regular PPI use ($p=0.019$).

This data shows that empiric PPI therapy titrated to control symptoms in the primary care setting results in an endoscopic progression to vCLO with and without intestinal metaplasia. Whether PPI therapy causes this conversion is unproven. However, the fact that PPI use does not prevent progression to vCLO is proven because regular PPI use had a significantly higher conversion rate than no PPI use.

This study shows, by performing endoscopy that would not have been recommended in most of these patients by the present treatment algorithm, that empiric PPI therapy for GORD results in the conversion of nearly 10 % of patients with symptomatic GORD to vCLO in 5 years. When one considers that 20–40 % of the population has symptomatic GORD, 10 % translates to an absolute number that easily explains why GORD-induced adenocarcinoma has increased sevenfold in the past four decades [5].

Definition of Irreversibility in GORD: vCLO

The best criterion for defining a significant point in any disease is the point where a pathologic change cannot be reliably reversed with any nonablative treatment. In GORD, at this point in time, that point of irreversibility is the occurrence of vCLO at endoscopy. In the United Kingdom, vCLO defines Barrett esophagus. In the USA and Europe, intestinal metaplasia is required for the diagnosis of Barrett esophagus. Medical treatment and antireflux surgery cannot reverse vCLO or reliably prevent its progression to intestinal metaplasia, increasing dysplasia and adenocarcinoma.

The definition of irreversibility in GORD has changed with the increasing effectiveness of acid suppressive therapy from 1950 onward with acid neutralizers, H_2 receptor antagonsists, and PPI coming into the market. Before this time, erosive esophagitis was irreversible, progressing to severe ulcers and strictures that were the major complications of GORD [6]. PPI therapy is highly effective in reversing erosive esophagitis [4]. In the Pro-GERD study, the number of patients with high-grade erosive esophagitis after 5 years of empiric therapy was 34/2481 (1.4 %).

However, at the same time as PPI therapy healed erosive esophagitis, it resulted in a nearly 10 % induction of vCLO in 5 years in the same patient population. The presently recognized end point of vCLO, which is esophageal adenocarcinoma, has replaced intractable ulcers and strictures as the main complication of GORD. In 1950, despite the fact that vCLO existed esophageal adenocarcinoma was rare; the first case was reported by Morson in 1952 [7].

The advantage with defining irreversible GORD by the presence of vCLO is that there is no established evidence that any patient who does not have vCLO progresses to adenocarcinoma. There can be argument about this. It can be argued that the person who is endoscopically normal that is found to have intestinal metaplasia at the normal squamocolumnar junction is at risk. However, present management guidelines recommend that patients who are endoscopically normal should not undergo biopsies because the risk of cancer in patients who have intestinal metaplasia is unknown [1]. The argument, therefore, has no practical merit at this time. It may change in the future if an increased cancer risk is defined in this group.

Unfortunately, the detection of vCLO requires endoscopy. In the Pro-GERD study, the only nonendoscopy findings that were significantly associated with progression to vCLO in the 5-year period were male gender, alcohol use, and regular PPI use.

If endoscopy is performed soon after the onset of symptoms without waiting for treatment failure, as was done in the Pro-GERD study, 240/2721 (8.8 %) patients would already have vCLO. In addition, the following endoscopic findings were predictive of progression to vCLO in 5 years: (a) presence of erosive esophagitis with risk increasing with grade of esophagitis; and (b) presence of intestinal metaplasia in a biopsy taken from the junction of an endoscopically normal patient. In another arm of the Pro-GERD study, patients who were endoscopically normal who had intestinal metaplasia at the squamo-columnar junction had a 25 % risk of progression to vCLO within 5 years [8].

If the presence of vCLO is recognized as the point of irreversibility in GORD, there can be *a new objective of management of the GORD patient,* i.e., *the prevention of progression to vCLO*. This would then provide an incentive for early endoscopy in the patient with GORD. Early endoscopy before failure with empiric treatment with PPI has the ability to recognize both irreversible GORD by the presence of vCLO and predict its occurrence within the next 5 years. There is a high probability that successful repair of the damaged lower esophageal sphincter (LOS) in the patient without vCLO has a high probability of preventing vCLO.

If we are successful in the objective of preventing vCLO, there is the strong likelihood that we will substantially prevent GORD-induced adenocarcinoma. Surely, this is a noble objective.

Cause of GORD: Lower Esophageal Sphincter Damage

The esophagus is a tubular structure that is approximately 25 cm long. It begins in the neck, traverses the posterior mediastinum, and passes through the diaphragmatic hiatus into the abdomen where it enters the stomach at the gastroesophageal junction (GOJ). In its normal resting state, it is closed at both ends by two sphincters.

When a food bolus enters the pharynx, the deglutition reflex is initiated, causing both sphincters to relax and a propagative peristaltic wave to develop. This propels the food bolus into the stomach. When the food enters the stomach, both sphincters regain their resting high pressure state.

The LOS acts as a barrier that prevents reflux of gastric contents into the esophagus [9, 10]. Its design is beautifully adapted to perform this function. The LOS pressure is normally >15 mmHg, exceeding the baseline luminal pressure in the esophagus (normally around −5 mmHg) proximally and the baseline luminal pressure in the stomach (normally around +5 mmHg) distally. The LOS therefore acts as a valve that effectively prevents reflux along the natural pressure gradient that exists from the stomach into the esophagus (Fig. 4.2).

The Normal LOS and Consequence of Abdominal LOS Damage

The functional state of the LOS can be defined manometrically by three separate components [9, 10]: its mean pressure, its total length, and the length of its abdominal segment. Manometric studies of "normal" subjects indicate that the "normal" LOS pressure is >15 mmHg, the total LOS length is 40–50 mm, and the length of the normal abdominal segment is 25–30 mm with some outliers.

Unfortunately, the LOS is not easy to define by pathologic study. While careful study of the smooth muscle in the region of the GOJ has identified arrangements of muscle fibers that could represent the LOS [11], routine pathologic study of

resected specimens and at autopsy cannot define the LOS either in its functional or damaged state.

Manometric definition of the LOS has two problems:

(a) The measurement is imprecise. Present high resolution manometry uses a catheter with pressure sensors placed at 10 mm intervals. This is accurate in defining mean pressure, but relatively imprecise in defining small changes in the length of the sphincter (Fig. 4.2). The older motorized pull-through manometry system, though less patient-friendly, provided more accurate data on length, but is rarely used today.

(b) Manometry only defines the functional LOS. When the LOS is damaged, it loses its resting high pressure. If this occurs at the distal end, the pressure in the damaged part of the abdominal segment of the LOS becomes equal to gastric luminal pressure and cannot be detected at manometry.

A largely unappreciated normal function of the abdominal segment of the LOS is to maintain the tubular shape of the abdominal esophagus by resisting the positive (around +5 mmHg) intraluminal pressure. When the abdominal LOS is damaged, the protection provided by the tonic contraction of the LOS is lost. The part of the

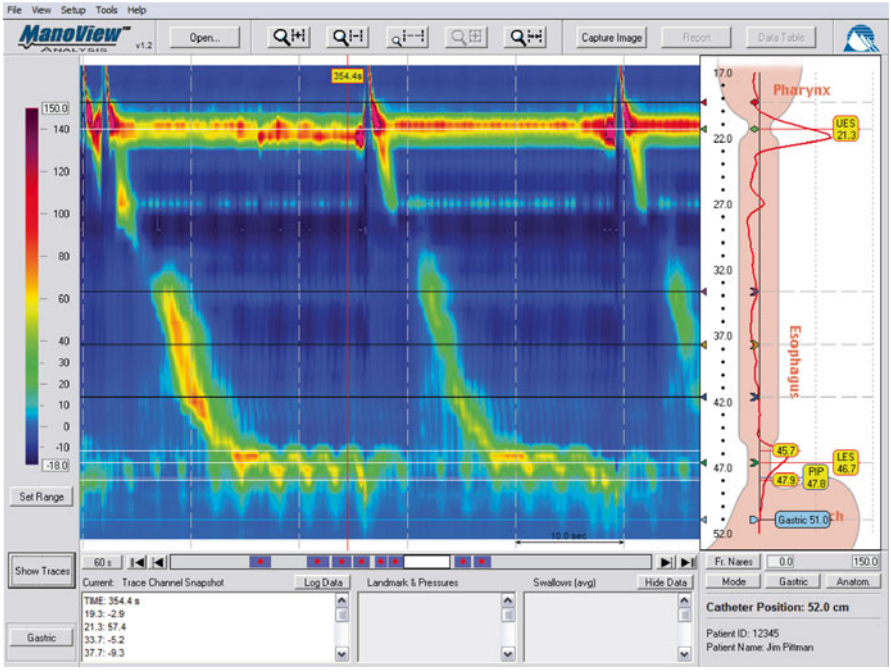

Fig. 4.2 High resolution manometry showing the esophageal pressure tracing during three swallows. The lower eosphageal sphincter relaxes during the swallow and regains its resting pressure between swallows. The pressures and ends of the sphincter are less precisely defined than in the stationary pull-through method

distal abdominal esophagus that has lost sphincter tone will be subject to the dilatory positive resting intraluminal pressure, which will be exacerbated during meals when the stomach distends and the intragastric pressure increases.

The distal abdominal esophagus that has lost LOS pressure will therefore dilate to form the dilated distal esophagus [12]. The tubular esophagus shortens, the damaged esophagus dilates and takes up the gastric contour and becomes part of the reservoir, and the angle of His becomes more obtuse. Mucosal rugal folds, which are a feature of all reservoir organs, develop in this dilated distal esophagus that results from loss of abdominal LOS function.

Damage to the abdominal LOS with loss of its resting pressure therefore results in "gastricization" of the distal esophagus to the length that the abdominal LOS is shortened. This gastricization occurs at a manometric, endoscopic, and gross anatomic level, leading to confusion that has created error in this region from the beginning of time and continues to the present [13]. The only modality that can solve this puzzle is the correct interpretation of histology of this region.

Mechanism of LOS Damage

LOS damage is the result of pressure exerted from below as a result of a heavy meal that causes gastric over-distension [14]. Robertson et al. [15] showed elegantly that gastric over-distension causes "taking up" or "effacement" of the distal part of the LOS into the gastric contour, resulting in a temporary decrease in LOS length. The squamous epithelium lining the effaced LOS is exposed to gastric juice because effacement of the LOS causes the pH transition point to move proximally (Fig. 4.3).

There is a pocket of strong acid at the height of the food column during a meal [16]. Repeated and frequent exposure of the squamous epithelium to this acid pocket during gastric over-distension during heavy meals results first in reversible injury to the distal esophageal squamous epithelium followed by permanent columnar metaplasia of the squamous epithelium.

If LOS damage occurs because of pressure from below, it must follow that LOS damage begins at its distal end and progresses upward. Loss of length therefore begins in the distal abdominal segment of the LOS. Robertson et al. [15] showed that early LOS shortening associated with asymptomatic volunteers with central obesity was entirely in the abdominal segment and did not affect the thoracic LOS.

GORD can therefore be considered to be basically the result of an eating disorder. Viewed in this light, each person can be regarded as having a unique relationship between his/her eating habit, the response of the LOS to this over-eating, and the damage caused to the esophageal squamous epithelium by exposure to gastric juice.

At one extreme, the patient's LOS is not damaged by the effect of his/her eating habit on the LOS. This patient does no damage to the LOS and never gets GORD. At the other extreme, the patient's LOS is damaged early in life by an excessive eating habit and progresses rapidly to LOS incompetence, severe reflux, and damage to the

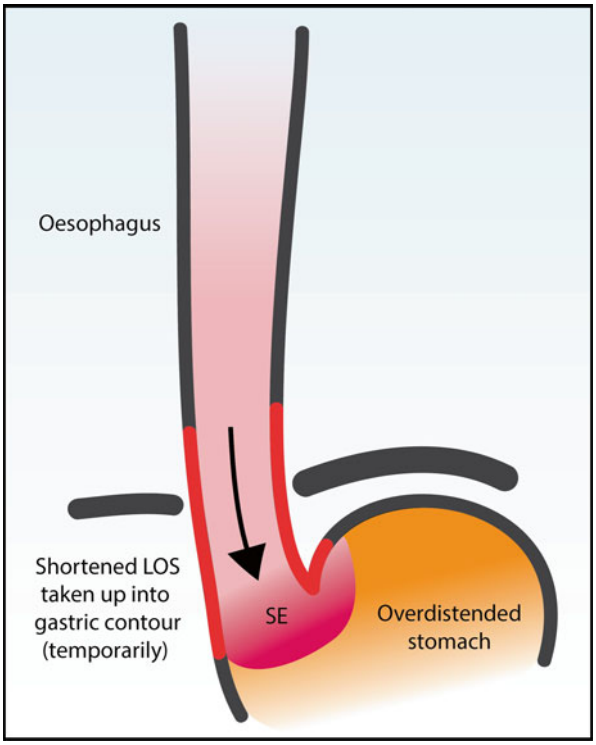

Fig. 4.3 Mechanism of exposure of the squamous epithelium of the distal esophagus to acid. When the stomach over-distends with a heavy meal, the LOS shortens, the distal LOS becomes effaced, i.e., moves down into the contour of the gastric fundus and the squamous epithelium becomes exposed to gastric contents of the full stomach. There is, at the top of the food column, an acid pocket that meets the descending squamous epithelium

squamous epithelium of the esophageal body at a relatively young age. This damage includes erosive esophagitis and becomes irreversible when vCLO occurs. Between these two extremes is the entire clinicopathologic spectrum of GORD. Progression of GORD can therefore be defined theoretically by the rate of progression of LOS damage resulting from a person's eating habit.

Relationship Between LOS Damage and GORD

While there is a certainty that failure of the LOS is the cause of GORD, there has never been any ability to correlate LOS damage with the severity of GORD. At present, manometry is a rarely used diagnostic test in the assessment of management of GORD until antireflux surgery is being considered. It is not useful in the diagnosis of early GORD.

Kahrilas et al. [17] demonstrated the close relationship of the LOS length to GORD. He measured baseline total LOS length in three groups with increasing severity of GORD: patients who had no GORD ("normal"), patients with GORD without a hiatal hernia, and patients with GORD who had a hiatal hernia. There was a significant shortening of baseline LOS length from normal to nonhernia GORD to hernia GORD. This correlated with an increase in baseline reflux as measured by a pH electrode placed 5 cm above the upper border of the LOS.

In this study, Kahrilas et al. [17] infused air into the stomach at 15 ml/min, causing progressive gastric distension. This caused an additional shortening of the LOS of 5–7 mm from baseline in all three groups as distension increased. The additional temporary shortening of the LOS was similar in the three groups, suggesting that gastric over-distension caused LOS exposure to damage in a linear manner. There was no vicious cycle phenomenon where a more damaged LOS was more susceptible to gastric over-distension. During the temporary shortening of the LOS with gastric distension, reflux episodes in the esophagus increased significantly and most prominently in the hernia-GORD group. This showed that a damaged LOS was more susceptible to failure when exposed to gastric distension.

The criteria of the LOS that correlate with the presence of sufficient reflux into the esophagus and clinical GORD are [10]: (a) a decrease in the mean LOS pressure to <6 mmHg, (b) a decrease in total LOS length to <20 mm, and (c) a decrease in abdominal length to <10 mm. At these levels of LOS damage, sphincter failure occurs so frequently that it results in an abnormal pH test and significant exposure of the squamous epithelium in the body of the esophagus to reflux. LOS damage defined by these criteria correlates with the presence of regurgitation, severe grades of erosive esophagitis, and vCLO.

There is a significant gap between the criteria that define a normal LOS and a defective LOS that is associated with abnormal reflux into the esophagus as defined by an abnormal pH test and the presence of clinical GORD. The mean LOS pressure must decrease from a normal of >15 to <6 mmHg; the total LOS length must decrease from a normal of 40–50 mm to <20 mm; and the abdominal LOS length must decrease from 25 to 30 mm to <10 mm before it becomes a criterion of LOS failure.

Part of this gap between normal and defective represents the reserve capacity of the LOS. As LOS damage increases, its reserve capacity is progressively reduced, but as long as it is not exhausted, the LOS maintains its competence (Table 4.1). This early LOS damage cannot be recognized by any present criterion for the diagnosis of GORD: the patient has no symptoms, endoscopic abnormality, or manometric criteria of a defective LOS or an abnormal pH test. This state where the LOS is damaged within its reserve functional capacity can be called "preclinical GORD." We will show that the histologic squamo-oxyntic gap can define and measure this early LOS damage.

The analogy of this progression of GORD is similar to the progression of ischemic heart disease. This is caused by progressively increasing coronary artery narrowing by atherosclerosis. In the 1960s, there was no clinical method of detecting coronary artery disease. Most patients had coronary atherosclerosis that progressed

Table 4.1 Length of abdominal segment of LOS, result of damage (shortening), and its correlation with LOS failure and severity of GORD

LOS(a) shortening	Residual LOS(a) length (mm)	LOS(a) length with a meal (mm)	Probability of LOS failure	Severity of GORD
Zero	25–30	15–30	Near zero	Zero
0–<5 mm	20–30	10–30	Near zero	Near zero
5–<10 mm	15–25	5–25	Postprandial only	Mild
10–<15 mm	10–20	0–20	Postprandial mainly	Moderate
>15 mm	<10–15	0–15	Frequent	Severe
>20 mm	<5–10	0–<10	Incessant	Very severe

The normal abdominal LOS measures 25–30 mm. Shortening <5 mm is within the reserve capacity of the LOS, which is still competent even with a 5–10 mm shortening associated with a heavy meal. At 5–15 mm shortening, postprandial reflux which can likely be controlled by modifying eating habits and/or PPI is likely. With greater LOS shortening, the likelihood of LOS failure progressively increases, resulting in increasing severity of reflux. The correlation of severity of reflux with symptoms is inexact; its relationship to erosive esophagitis and Barrett esophagus is stronger. Patients with LOS damage >15 mm are those at greatest risk for failing medical therapy, progressing to visible columnar lined esophagus and adenocarcinoma
LOS(a) = abdominal segment of the lower esophageal sphincter
Residual LOS length = Original LOS(a) length — LOS(a) shortening
LOS(a) length with a = temporary LOS(a) shortening of 5–10 mm with a heavy meal. There is no shortening with a meal that causes no gastric overdistention
Probability of LOS failure increases as the LOS(a) length decreases to <10 mm
Severity of GORD = frequency of LOS failure
Postprandial LOS failure can be controlled by dietary modification that can control that amount of LOS(a) shortening associated with a meal between 0 and 10 mm

slowly and remained within the reserve capacity of the vessels without causing ischemic heart disease. A few narrowed their vessels sufficiently to cause angina of effort. Some progressed to severe ischemic heart disease with myocardial infarction and death. Preclinical LOS damage, clinical GORD, and progression to vCLO and adenocarcinoma have an eerie similarity to this progression.

PPI therapy is to GORD what nitroglycerin was to ischemic heart disease: a method of controlling symptoms without addressing the cause of a disease that was present in most people, but that progressed to severe disease and a fatal outcome in a significant minority of patients. In ischemic heart disease, this was myocardial infarction. In GORD, it is Barrett esophagus and esophageal adenocarcinoma.

There is one important difference. Coronary atherosclerosis was easily detectable at autopsy. The relationship between coronary artery narrowing and ischemic heart disease was easily correlated in autopsy studies. All that was needed was for technology to advance to permit clinical detection of coronary arterial disease. In contrast, LOS damage cannot be detected at autopsy by presently accepted criteria of histologic normalcy of this region. The presence of vCLO and adenocarcinoma cannot be attributed at autopsy to a defective LOS.

We will show that the correct use of histology permits definition of LOS damage with exquisite accuracy if interpreted correctly. When this is recognized, we will

have a method of study of GORD that is similar to that which propelled advances in the management of ischemic heart disease. GORD will be defined by its cause, LOS damage, defined by histology at endoscopy with appropriate specimens.

We hope that from this platform will emerge a method of scientific study of GORD based on its etiology. The technology needed exists today. It is simply not used or, when used, completely misunderstood. Once the early diagnostic criteria of LOS damage that predict progression to vCLO are defined, it will hopefully become a relatively easy step to identify patients destined to progress to vCLO and intervene early in these patients to prevent this progression.

Mechanism of Maintenance of LOS Pressure

The high pressure in the LOS is maintained by the tonic contraction of the smooth muscle in the entire length of the LOS. There is evidence that this is a function of the smooth muscle of the esophagus that is largely controlled by the intrinsic nerves. The tone of the LOS is retained even when the external neural connections of the esophagus are completely divided. However, destruction of the intrinsic neural fibers by administration of a neurotoxin results in loss of LOS tone, suggesting the existence of a local neuromuscular reflex mechanism for maintenance of LOS pressure.

There is limited data about the intrinsic neuromuscular connections in the esophagus. Rodrigo et al. [18], in an elegant study of the innervation of the normal squamous epithelium of the esophagus, showed the presence of afferent nerve endings at varying depths of the epithelium. These traversed the basement membrane of the epithelium and connected with a subepithelial nerve plexus that was derived from submucosal ganglion cells. The connections between the submucosal and myenteric plexus ganglion cells are poorly understood. It is believed that the effector fibers of the myenteric plexus play a role in normal smooth muscle contraction in the esophagus. This likely includes peristaltic muscle contraction in the esophageal body and the tonic contraction and relaxation of LOS muscle.

Elsewhere in the body, tone in skeletal muscle is maintained by local reflex arcs. Afferents from stretch receptors in tendon and muscle relay information to the alpha and gamma motor neurons in the spinal cord that produce muscle contraction that maintains normal tone. Loss of tone results when this reflex arc is interrupted in lower motor neuron lesions. Upper motor neuron and extrapyramidal lesions can increase muscle tone. If there is an analogous mechanism responsible for maintaining LOS tone, it would explain loss of tone when the local reflex is interrupted.

Possible afferents for such a local reflex arc include yet undiscovered receptors in the smooth muscle and the documented intraepidermal nerve endings in the squamous epithelium. If the afferent arm of the reflex arc is dependent on afferents nerve endings in the squamous epithelium, the occurrence of columnar metaplasia will necessarily interrupt the reflex arc and result in loss of LOS pressure.

In this chapter, we will explore the relationship of columnar metaplasia of the squamous epithelium in the LOS region and loss of LOS pressure. We will provide evidence that the length of columnar metaplasia of LOS squamous epithelium is concordant with the degree of shortening of the LOS. This suggests that replacement of squamous by columnar epithelium possibly changes the afferent input from the epithelium. Our goal is to use this relationship to introduce a potential new test that provides a measure of LOS shortening using targeted endoscopic mucosal biopsies.

In Search of Accurate Definitions

Precise and accurate definition is critical in forming a basis for scientific study. Precision requires simplicity and ease of application of the definition such that there is the lowest possibility of interobserver variation.

The present scientific study of the esophagus, and particularly the changes in the esophagus that occur when the esophagus is exposed to gastric acid, is compromised by lack of precise and accurate definition of many things that are critical. It is almost as if our faith that the treatment of GORD with PPI would make GORD be a disease of the past has prevented us from scientific study of GORD.

In fact, even the definition of gastroesophageal reflux disease (GORD) itself, lacks precision. The Montreal consensus definition states [19]: *"GORD is a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications."* This definition suggests that pathologic changes resulting from GORD cannot occur without reflux of gastric juice into the esophagus. Is this really true? What if the esophageal mucosa becomes exposed to gastric juice and undergoes damage without reflux from the stomach to the esophagus? Is this not also the equivalent of GORD? The mechanism of exposure of esophageal squamous epithelium to gastric juice is surely less important than the fact of that exposure.

We suggest that a more precise definition of GORD is that *"GORD is a pathologic condition resulting from exposure of the esophageal epithelium to gastric juice."* Converting the basis of definition from its dependency on relatively nonspecific and insensitive symptoms to a histologic basis is a positive step if accurate histologic changes of GORD can be defined.

This would mean that GORD can occur without any symptoms or abnormality in the tests that are commonly used for measuring reflux of gastric juice into the esophagus. These tests, which include pH and impedance testing, have the basic flaw that the measuring device is placed high in the esophagus, usually 5 cm above the upper end of the lower esophageal sphincter (LOS). Given a mean LOS length of 4–5 cm, the measuring device is 9–10 cm above the end of the esophagus. Using the presence of reflux by these tests is surely at risk of missing early changes of GORD. In fact, we know this to be true. There are patients with troublesome symptoms of GORD who have a normal pH or impedance test.

From an anatomical standpoint, it is very important to have a precise and accurate definition of the gastroesophageal junction (GOJ). The most widely used definition of the GOJ is the proximal limit of rugal folds [1, 3, 20]. This is a reasonably precise endoscopic landmark and can usually be seen in gross specimens. However, there is absolutely no evidence that it accurately represents the GOJ. The basis of the definition is the opinion of experts [3, 21]. For an opinion-based definition for which evidence is lacking, this definition of the GOJ has powerful international acceptance.

The absence of an evidence base for the universally accepted definition of the GOJ raises the question as to whether all interpretations that are accepted about the findings in this region are correct. We suggest that errors resulting from incorrect definition of the GOJ are indeed the reason why GORD is such a poorly managed disease [22].

In this chapter, we will develop a precise definition of the GOJ. When this is done, GORD changes from being a confusing entity to one that can be accurately defined with micrometer precision and complete understanding of its pathophysiology.

Histologic Definitions of Epithelia in the Esophagus and Stomach

It is remarkable that GORD, a disease that results from damage to esophageal epithelium by gastric acid, has no histopathologic criteria that have practical value in present diagnosis. Squamous epithelial changes that include dilated intercellular spaces, basal cell hyperplasia, papillary elongation, and intraepithelial eosinophils have such low specificity and sensitivity to have no predictive value. A significant number of patients with clinically "proven" symptomatic GORD are normal by present endoscopic and histologic criteria. These patients are designated as having nonerosive reflux disease (NERD).

Before we accept the fact that histology does not play a role in the diagnosis of early GORD, it is important to ask the right questions: Is there any possibility that we are overlooking something obvious? Is there some histologic change that is diagnostic of GORD that we are missing? Are we looking at the right things? Is there any possibility of error in our definitions? Could we be calling the distal esophagus damaged by GORD something else? Like the proximal stomach? Is it possible that we are so wrong? The simple answer to all these questions is a vehement "yes."

To begin to answer these questions and explore histologic criteria for defining early GORD, it is important to first define the epithelial types seen in the esophagus and stomach. There are only five epithelial types that occur from the proximal end of the esophagus to the pyloric antrum [23, 24]. These are as follows:

(a) Stratified squamous epithelium. This is limited to the esophagus in the human. It is *always* present.

(b) Gastric oxyntic mucosa. This is limited to the proximal stomach and not found in the esophagus. It is *always* present.
(c) Three columnar epithelial types that are *not always* present. When present, however, they are *always* interposed between the distal limit of esophageal squamous epithelium and the proximal limit of gastric oxyntic epithelium. These are (1) cardiac epithelium composed of only mucous cells; (2) cardiac epithelium with parietal cells admixed with mucous cells in the glands, an epithelium that we have designated as oxyntocardiac epithelium; and (3) cardiac epithelium with goblet cells, which we call esophageal intestinal epithelium. The prevalence of these three columnar epithelial types is variable. Intestinal epithelium is the least common and oxyntocardiac epithelium the most prevalent.

The four columnar epithelial types can be precisely defined by simple histologic criteria based on the presence or absence of mucous cells, parietal cells, and goblet cells (Table 4.2). The definition of the epithelial type is applied to every unit of the epithelium, which is defined as a single foveolar-gland complex. Multiple epithelial types can therefore be present in a small area.

The diagnosis of these four epithelial types has high precision with minimal training and experience. More important than training though is an understanding in the pathologist that differentiating between these epithelial types indeed has merit.

Careful study of routine sections stained by hematoxylin and eosin is adequate for accurate diagnosis in most cases. The only recommended special stain is digested periodic acid Schiff stain (diastase-PAS or D-PAS) if there is a question between oxyntocardiac and gastric oxyntic epithelium. D-PAS highlights neutral mucin containing mucous cells in the glands and facilitates differentiation of gastric oxyntic epithelium from oxyntocardiac epithelium with a predominance of parietal cells in the glands (Fig. 4.4). Alcian blue stain is strongly discouraged. Intestinal epithelium is defined by the presence of goblet cells visible on routine stained sections. It is not

Table 4.2 Histologic criteria for diagnosis of four different columnar epithelial types that are encountered in the esophagus and proximal stomach

	Mucous cells in glands[a]	Parietal cells	Goblet cells
Gastric oxyntic epithelium	–	+	–[b]
Cardiac epithelium	+	–	–
Oxyntocardiac epithelium	+	+	–
Intestinal epithelium	+	–	+

Gastric oxyntic epithelium lined the entire proximal stomach. Cardiac, oxyntocardiac, and intestinal epithelia are, when present, interposed between the squamous epithelium and gastric oxyntic epithelium (i.e., form the squamo-oxyntic gap)

Note: There is no epithelium defined in this scheme that has both parietal and goblet cells in one foveolar-gland complex. This is an extremely rare finding; when found, goblet cells take precedence and the epithelium is designated as intestinal

[a]Mucous cells are present at the surface and foveolar pit in all epithelial types; it is the presence of mucous cells in glands below the foveolar pit that are relevant to the definitions

[b]Gastric oxyntic epithelium with atrophic gastritis can have goblet cells. This is gastric intestinal metaplasia and different than cardiac epithelium with intestinal metaplasia

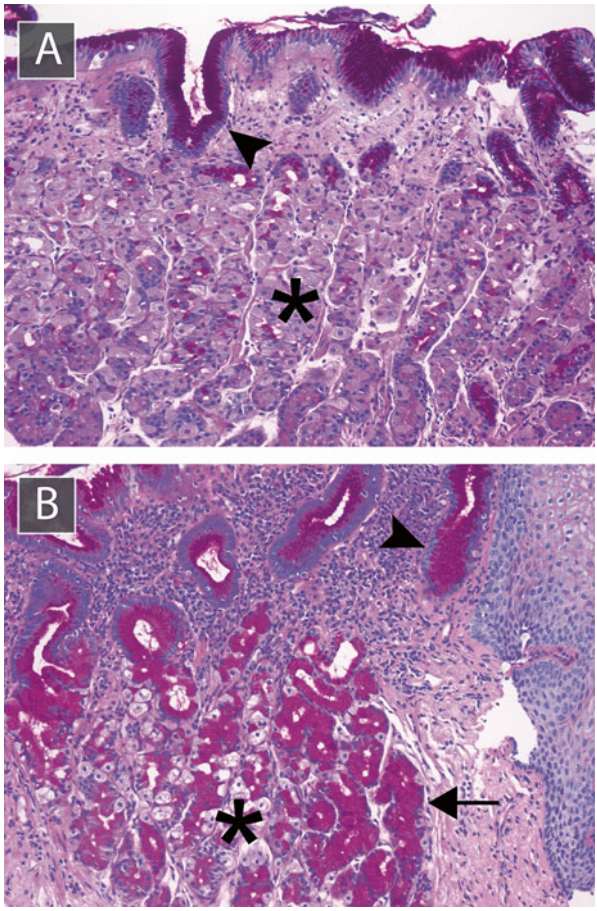

Fig. 4.4 D-PAS stain aids in the distinction of gastric oxyntic epithelium (**a**) where the positive deep magenta staining of mucous cells is limited to the surface foveolar region (*arrowhead*). The glands below the foveolar pit are PAS negative (*asterisk*). (**b**) Oxyntocardiac epithelium shows an admixture of mucous cells (*deep magenta, arrow*) round parietal cells (negative for PAS, *asterisk*) in the glands under the foveolar pit (*arrowhead*)

defined as the presence of blue staining acid mucin, which can be found in cardiac epithelial cells. The use of alcian blue stain has a high risk of overdiagnosis of intestinal metaplasia.

The Squamo-Oxyntic Gap

The squamo-oxyntic gap is a new concept that we introduced in 1997 and named in 2010 [25, 26]. Most pathologists and gastroenterologists do not use the concept despite the strong evidence that exists that it has value in the diagnosis of GORD [22]. The relatively small number of groups that have embraced the concept have produced data that have uniformly shown that the presence of a squamo-oxyntic gap correlates with the presence of GORD, its length with the severity of GORD, and its composition with the risk of adenocarcinoma.

The squamo-oxyntic gap is defined histologically. Its relationship to the tube and pouch, as well as its relationship to the rugal folds in the region of the GOJ is of secondary relevance. It must be used without preconceived ideas of anatomy and the recognition that these may be incorrect [13].

The best way to assess the squamo-oxyntic gap is by a vertical section taken with its proximal end at the squamocolumnar junction extending distally till the proximal limit of gastric oxyntic epithelium is reached. In resection specimens and autopsies, this vertical section should extend 30 mm beyond the end of the tube to ensure that gastric oxyntic epithelium is reached.

Its length can be assessed at endoscopy by measured biopsies taken from the squamocolumnar junction, extending distally to a point 30 mm distal to the point of flaring of the esophagus. For practical purposes, 15–20 mm is sufficient; to be thorough, 30 mm is optimal although it is likely that all biopsies taken 30 mm distal to the end of the tube will be composed of gastric oxyntic epithelium. The intervals of the measured biopsies can be 5 or 10 mm with the understanding that precision of measurement increases as the biopsy interval decreases. Ideally, someone should develop a new biopsy instrument that can obtain a 20 mm vertical biopsy of the mucosa. This will provide a measurement that is accurate at a micron level and identical to a vertical section taken from a resection specimen.

A study of the variations in the length and composition of the squamo-oxyntic gap and finding reasons for these variations provides a unique method of elucidating the pathologic changes of GORD. The study of the relationship of the squamo-oxyntic gap to endoscopic and gross anatomic landmarks permits accurate definition of the GOJ and removes the existing false dogmas that cloud the study of GORD at the present time.

Definition of the Squamo-Oxyntic Gap

Because cardiac, oxyntocardiac, and intestinal metaplastic epithelia are *always* found between the distal end of the squamous epithelium and the proximal limit of gastric oxyntic epithelium, it is possible to define a histologic entity called the squamo-oxyntic gap. This is the gap between the distal limit of squamous epithelium (always esophageal) and the proximal limit of gastric oxyntic epithelium (always gastric).

The ability to define and measure the squamo-oxyntic gap provides a histologic springboard for the scientific study of the esophagus and proximal stomach. Its definition is easy, precise, and without any controversy. No one can argue with the fact that the gap lies between squamous epithelium and gastric oxyntic epithelium.

Care must be taken when evaluating the literature to recognize that there are two different gaps in this region based on criteria used for definition:

(a) The squamo-oxyntic gap [26–28], as defined earlier, which includes oxyntocardiac epithelium and cardiac epithelium with and without intestinal metaplasia;

(b) The gap between the distal limit of squamous epithelium and the first parietal cell that is encountered [15, 29]. This is NOT the squamo-oxyntic gap. It is the length of cardiac epithelium. This is always less than the squamo-oxyntic gap because oxyntocardiac epithelium is invariably present distal to cardiac epithelium in patients who have cardiac epithelium. The measured length of the gap between the distal limit of squamous epithelium to the first parietal cell (i.e., the length of cardiac epithelium) does not bear a constant relationship to the length of the squamo-oxyntic gap. As such, it cannot be used to calculate the total length of the squamo-oxyntic gap.

The Length of the Squamo-Oxyntic Gap

The length of the squamo-oxyntic gap varies in published reports from zero to over 20 mm (Fig. 4.5). A gap of less than 2 cm is usually not recognized at endoscopy. A gap exceeding 3 cm is recognized as vCLO at endoscopy. Visible CLO at endoscopy, when present, is always significantly shorter than the total length of the histologic squamo-oxyntic gap, usually by 10–25 mm.

There is a present controversy as to the normal length of the gap. We believe that *the normal gap is zero*. It is easy to establish this positive. We [27], and other groups [30], have illustrated a squamocolumnar junction with a direct transition of squamous to gastric oxyntic epithelium without a gap (Fig. 4.5). If a zero squamo-oxyntic gap is present in anyone without any detectable abnormality, this must be the normal state. We have also seen a near zero squamo-oxyntic gap in an esophagectomy specimen of a 77-year-old patient with squamous carcinoma.

By our viewpoint, any squamo-oxyntic gap is abnormal and results from columnar metaplasia of esophageal squamous epithelium caused by exposure to gastric juice, i.e. GORD. Glickman et al. [31] showed that pediatric patients who had

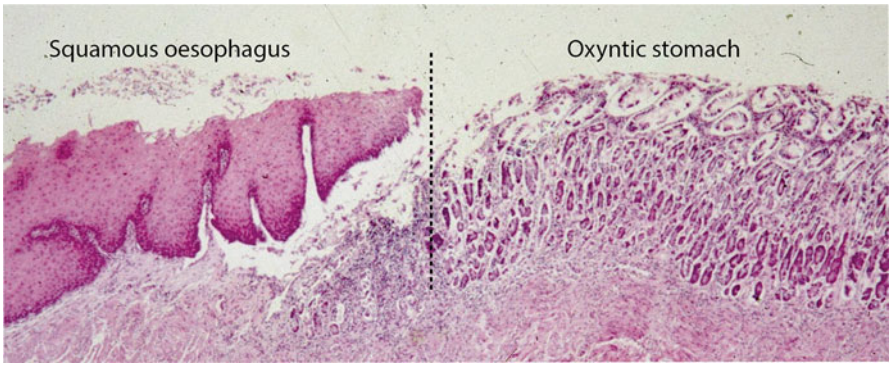

Fig. 4.5 A section across the squamocolumnar junction at autopsy showing direct transition of squamous epithelium to gastric oxyntic epithelium, characterized by the typical straight tubular glands containing only parietal and chief cells below the foveolar pit

>1 mm of cardiac epithelium distal to the squamocolumnar junction had more reflux than children with <1 mm. Increasing length of the gap correlates very well with increasing severity of GORD. Chandrasoma et al. [32] showed that patients with >20 mm of a squamo-oxyntic gap had more reflux than those with <20 mm.

The opposing viewpoint is that a gap of "up to 4 mm" composed of cardiac epithelium normally exists in the proximal stomach ("the gastric cardia") [29, 33]. This is not a tenable viewpoint with data showing that 1 mm of cardiac epithelium correlates with GORD in children. There is also no pathologic entity at the present time that explains why the gap can extend up to 30 mm into what is called the proximal stomach, 26 mm greater than what is deemed normal. The 1–4 mm normal cardiac mucosa in the proximal stomach is associated with GORD and its expansion into 30 mm is associated with more severe GORD. The belief presently held by most pathologists and gastroenterologists of the concept of a "normal squamo-oxyntic gap" consisting of "normal cardiac epithelium" in the proximal stomach ("gastric cardia") is dying, albeit very slowly.

When defining normalcy, it is an error to equate normalcy with universality. A finding that is present in everyone is not necessarily normal. For example, atherosclerosis of the abdominal aorta is present in almost all people in the Western world. The reason why this is not normal is that a person with atherosclerosis is more likely to develop ischemic vascular disease than one without. Similarly, the presence of a squamo-oxyntic gap is not "normal," because its presence is more likely to indicate GORD than its absence.

The Epithelial Composition of the Squamo-Oxyntic Gap

The epithelial composition of the gap varies greatly. All patients with a gap will have oxyntocardiac epithelium at the distal end of the gap. The majority of people with a gap length of <5 mm have only oxyntocardiac epithelium separating squamous from gastric oxyntic epithelium [27].

The majority of patients with a gap length >1 cm will have a mixture of cardiac and oxyntocardiac epithelium [34]. Intestinal metaplasia is present in a minority (5–10 %) of people. Its prevalence increases with increasing length of the squamo-oxyntic gap [34]. At the present time, nearly 100 % of patients with a gap length >5 cm will have intestinal metaplasia [34].

Surprisingly, the distribution of the three epithelial types, when all are present, is remarkably constant [35]: intestinal metaplasia is found proximally adjacent to squamous epithelium, oxyntocardiac is found distally adjacent to gastric oxyntic epithelium, and cardiac epithelium is found in the intermediate zone by itself and admixed with the other two epithelia proximally and distally.

The prevalence of intestinal metaplasia increases with increasing length of the gap and it is predominantly present in the more proximal region of the gap. The only logical explanation for these findings is that the occurrence of intestinal metaplasia in cardiac epithelium is favored by the more alkaline environment of the

more proximal esophagus in a patient with GORD. This would explain the dramatic increase in Barrett esophagus with intestinal metaplasia in the past four decades. Acid suppressive drug therapy has resulted in increasingly effective alkalinization of gastric juice and therefore the esophagus during a reflux episode. Modern PPI therapy can maintain gastric pH at >4 for >18 h of the day [36].

The Location of the Squamo-Oxyntic Gap

Localization of the gap is entirely dependent on the definition of the GOJ. Error in the definition of the GOJ will result in incorrect localization of the gap. The fact that the presently used definitions of the GOJ (the point of flaring of the tubular esophagus and proximal limit of rugal folds) has no evidence base to support them [1, 3, 21], should make us keep an open mind to the possibility that they are inaccurate. The reported location of the squamo-oxyntic gap varies from purely esophageal to purely gastric, to straddling the GOJ and involving both esophagus and proximal stomach. In the literature, the squamo-oxyntic gap extends into what is called the proximal stomach to a maximum of 30 mm. By presently accepted criteria, this is the "gastric cardia."

It is remarkable that the original description of the squamo-oxyntic gap by Allison and Johnstone in 1953 [37] was the same as the concept that we have developed with much sweat and controversy over the past two decades. Allison and Johnstone's perspective was based on esophago-gastrectomy specimens [37]. At the time, the GOJ was defined externally by the point of transition of the esophageal muscle to gastric muscle and the location of the peritoneal reflection. There were no internal definitions of the GEJ. The pathologist, Dr. D. H. Collins, who reported the best described case, divided the specimen horizontally at the peritoneal reflection, essentially separating the stomach from the esophagus at the GOJ as defined in 1953. He then took a long vertical section that traversed the entire specimen for histologic examination. There was no need to differentiate tube from sac or to define the rugal folds; these were irrelevant at the time. The epithelium at and distal to the peritoneal reflection was gastric oxyntic epithelium. The epithelium proximal to the GOJ was cardiac epithelium with scattered parietal cells. Two of eleven cases had goblet cells; there was one patient with adenocarcinoma. No gastric oxyntic epithelium was present in the esophagus.

By the definitions of the GOJ used in 1953, the entire squamo-oxyntic gap was located in the esophagus with its distal end at the GOJ. The stomach distal to the GOJ was lined by gastric oxyntic epithelium [37].

The increasing use of endoscopy in the next decades created the need for definition of the GOJ from the mucosal aspect. The external markers of the GEJ used by Allison and Johnstone in resected specimens were not visible at endoscopy. The landmarks that can be identified with reasonable precision at endoscopy are the squamocolumnar junction (Z-line), the diaphragmatic impression, the point of flaring of the tubular esophagus, the distal end of the palisading longitudinal vessels,

and the proximal limit of rugal folds. While recognizable as landmarks, none of these have been shown to correspond accurately to the GOJ in both normal and GORD-damaged esophagus.

A good example of the desire to use a mucosal landmark to define the GOJ is Norman Barrett's statement in 1950 [38]: *"the oesophagus is that part of the foregut, distal to the cricopharyngeal sphincter, which is lined by squamous epithelium…"* Barrett incorrectly defined the GOJ as the squamocolumnar junction. By Barrett's original definition, the entire squamo-oxyntic gap in the tubular esophagus was gastric and called the "tubular intra-thoracic stomach". Barrett, in 1957 [39], changed his mind when he agreed with Allison and Johnstone that what he had called the tubular intrathoracic stomach was esophagus, an entity which he named the columnar lined esophagus that bears his name.

In 1961, Hayward [40] defined the GOJ as the end of the tube where it flared into the saccular stomach. He gave no reason or evidence to support this except to opine that the function of the esophagus is to transmit food from the pharynx to the stomach and this function must require a tube. Using this definition of the GOJ, Hayward defined the normal histology of this region. According to this, there was normally a squamo-oxyntic gap of approximately 5 cm [40]. The normal squamocolumnar junction was 2 cm proximal to the end of the tube. Cardiac epithelium extended from that point across the GOJ (the end of the tube) into what he called the proximal stomach to a distance of 3 cm before gastric oxyntic epithelium was encountered. According to Hayward, this 5 cm of a mucous cell containing epithelium acted as a buffer that prevented acid digestion of the esophageal squamous epithelium. Hayward produced no data to justify his edicts. These definitions of the GOJ and the 5 cm normal extent of cardiac epithelium that was found both in the distal esophagus and proximal stomach were accepted by the entire pathology and gastroenterology world for the next 3 decades. Five centimeters of a squamo-oxyntic gap had, by the power of Hayward's edict, become a normal structure located in both distal esophagus and proximal stomach [40].

In 1994, Spechler et al. [2] provided data that led to the definition of short-segment Barrett esophagus. This resulted in the recognition that the concept that columnar epithelium normally lined the distal 2–3 cm of the esophagus was not correct. The endoscopic definition of the normal state was that squamous epithelium extended all the way to the end of the tube where the horizontal squamocolumnar junction met the proximal limit of rugal folds. The normal squamo-oxyntic gap had now become limited to the proximal 3 cm of the stomach.

Autopsy Studies of the GOJ

All the studies that had produced data regarding the normal structure of the GOJ came from the study of resection specimens and endoscopy in patients with GORD. The first detailed autopsy studies of the GOJ in patients who had no clinical

evidence of GERD were published in 2000 from our unit [27]. This presented a new perspective that was remarkably different than the accepted dogma of normal histology in this region. Complete circumferential histologic examination of the 2 cm distal to a grossly normal squamocolumnar junction in 18 patients revealed the following [27]:

(a) A squamo-oxyntic gap was present in all patients but not in the entire circumference of the squamocolumnar junction. In 50 % of patients, there was a direct transition from esophageal squamous epithelium to gastric oxyntic epithelium in some part of the circumference (Fig. 4.5).
(b) Intestinal epithelium was not present in any patient.
(c) Cardiac epithelium was absent in 56 % of patients.
(d) Oxyntocardiac epithelium was present in some part of the circumference in all patients.
(e) The length of the squamo-oxyntic gap ranged from a minimum of zero to a maximum of 0.805 cm in the 18 patients.
(f) The person with the shortest gap had only oxyntocardiac epithelium to an extent of zero to 0.048 cm.

In an almost simultaneous study of pediatric autopsies in 2000, Kilgore et al. [29] confirmed that cardiac epithelium was much shorter than previously thought (0.1–0.4 cm) but found it to be present in all patients. Kilgore et al. did not report the length of oxyntocardiac epithelium. It is well established that cardiac epithelium can be present more than 2 cm distal to a normal squamocolumnar junction in patients with GORD [28, 41]. The critical information in these autopsy studies relates to the minimum, not maximum extent of cardiac epithelium. This is zero in the Chandrasoma et al. [27] study and 0.1 cm in the Kilgore et al. study [29]. The common interpretation of these two studies in the pathology and gastroenterology literature was that cardiac epithelium was normally present "up to 0.4 cm" as a normal epithelium in the proximal stomach [33]. This erroneous conclusion has been repeated as fact by all major authorities despite the fact that the conclusion is obviously flawed [13, 22].

Until this misconception that the most distal 1–4 mm of the squamo-oxyntic gap is normal gastric epithelium is corrected, the argument will always be whether 1–4 mm of cardiac epithelium is a normal proximal gastric epithelium in addition to being the epithelium that constitutes vCLO. Derakhshan et al. recently showed that the <2.5 mm of cardiac epithelium found in asymptomatic volunteers showed inflammation and immunohistochemical features that were similar to nonintestinalized vCLO [42]. Spechler, in an editorial opinion of this paper suggested that this data provided strong support for our concept that cardiac epithelium is *always* a metaplastic epithelium resulting from squamous epithelial damage caused by exposure to gastric juice.

The Squamo-Oxyntic Gap in Different Populations

The length of the squamo-oxyntic gap correlates with the severity of GORD in a manner that is highly predictable.

People Without GORD Symptoms

People without symptoms of GORD are reported in (a) autopsy studies (see earlier), (b) studies of asymptomatic volunteers, and (c) screening upper endoscopy. In autopsy studies, the squamo-oxyntic gap is <5 mm in the majority and range from zero to 10 mm [27, 29]. Robertson et al. [15], in a study of asymptomatic volunteers, measured the length of cardiac epithelium distal to the normal squamo-columnar junction in two groups of patients defined by the presence and absence of central obesity. Taking biopsies across the squamocolumnar junction with jumbo biopsy forceps permitted measurement of the length of cardiac epithelium distal to the junction. Cardiac mucosa was significantly longer in persons with central obesity compared to those without (2.5 mm vs. 1.75 mm). This is not the squamo-oxyntic gap because oxyntocardiac epithelium is excluded, but it shows that asymptomatic persons have cardiac epithelial lengths that are similar to those at autopsy.

Unfortunately, there is almost no other screening data in asymptomatic people. There is an opportunity to study the squamo-oxyntic gap at every upper endoscopy that is performed. Unfortunately, present understanding of GORD and the histopathology of this region has resulted in the general recommendation by all gastroenterology societies that biopsies should not be taken in the person who is endoscopically normal, whether or not they have symptoms of GORD [1, 3, 21].

GORD Patients with No Endoscopic Abnormality

There are very few studies that have reported systematic biopsies taken distal to a normal squamo-columnar junction that permit estimation of the length of the squamo-oxyntic gap. Even fewer studies provide data that allow correlation between the estimated length of the gap with evidence of GORD. Jain et al. [41] performed multilevel biopsies distal to the squamocolumnar junction in 31 patients. In 25 patients, the squamocolumnar junction was at the same level as the endoscopic GOJ; in the other six, it was >10 mm proximal to the GOJ. Four-quadrant biopsies were taken at the GOJ and 10 and 20 mm distally. The prevalence of cardiac epithelium was 11/31 (35 %) at the GOJ, 4/29 (14 %) 10 mm distal, and 1/30 (3 %) 20 mm distal. This data suggests that the cardiac epithelial length was >10 mm in 14 % and

>20 mm in 3 %. The authors did not report the presence of oxyntocardiac epithelium. As such the length of the entire squamo-oxyntic gap is unknown. Ringhofer et al. [43] reported the findings of multilevel biopsies at the endoscopic GOJ and on both sides of it at intervals of 0.5 cm. The level of the proximal limit of rugal folds was designated 0 and biopsies proximal to this were labeled +0.5 and those distal to it were designated −0.5, −1.0. All patients had a squamo-oxyntic gap. In no patient did squamous mucosa transition directly to gastric oxyntic mucosa. The gap consisted of only oxyntocardiac epithelium in 12 (11.8 %) patients, cardiac and oxyntocardiac epithelium in 71 (69.6 %) patients, and had intestinal metaplasia in 19 (18.6) patients. In 81 % of patients, the squamo-oxyntic gap extended to the biopsy taken 5 mm distal to the GOJ and in 28 % the gap extended to the biopsy taken10 mm distal to the GOJ. The relationship of the gap length to any criterion of severity of GORD was not reported.

The earlier studies show that, in patients with GORD, the squamo-oxyntic gap extends distal to the end of the tubular esophagus to a distance that is very commonly >5 mm, commonly >10 mm, and rarely >20 mm. The squamo-oxyntic gap is longer in patients with GORD than in people without symptoms of GORD at autopsy [27, 29] or study in asymptomatic volunteers [15]. This variation in length is easily measured by multilevel biopsies at endoscopy. It is never done. To most gastroenterologists, the gap is irrelevant. Being distal to the endoscopic GOJ, they define this as “normal” epithelium of the gastric cardia.

There are very few studies that report the correlation between the presence of a squamo-oxyntic gap and evidence of GORD. Glickman et al. [31], in a study of children who had a biopsy that straddled the squamocolumnar junction, measured the gap between the distal limit of the squamous epithelium and the first parietal cell, i.e. the length of cardiac epithelium (not the squamo-oxyntic gap). The showed that 52 % of children with a length of cardiac epithelium >1 mm had evidence of esophagitis, significantly more than 21 % of children who had a cardiac epithelial length <1 mm. This study proves that, in children, the presence of cardiac epithelium has a quantitative association with GORD at the miniscule length of 1 mm. Any cardiac epithelium in this region indicates columnar metaplasia resulting from squamous epithelium damage from exposure to gastric juice. Oberg et al. [44], from our unit, reported 334 patients with no endoscopic abnormality. A 2–4 sample retroflex biopsy was routinely taken within 10 mm from the squamocolumnar junction. Two-hundred and forty-six (74 %) had cardiac and/or oxyntocardiac epithelium; 88 (26 %) had neither of these epithelia. There was no exact measurement of the gap. However, it is highly likely that the 88 patients had a gap that was likely to be <10 mm and the 246 patients a gap >5 mm. The 246 patients had significantly higher 24-h pH study (6.0 vs. 1.1), lower mean LOS pressure (8.0 vs. 15.2), shorter length of abdominal segment of the LOS (10 vs. 16 mm), and higher percentage of a defective LOS (62.3 vs. 27.2) [44].

Patients with a vCLO at Endoscopy

Endoscopically visible CLO is universally accepted as being caused by GORD-induced metaplasia of the squamous epithelium of the esophagus. The change is absolutely specific for GORD; it is not seen in any other esophageal disease. Endoscopic biopsies taken at the proximally displaced squamocolumnar junction always show cardiac epithelium with or without intestinal metaplasia [34]. In these patients, the distal end of the columnar lined segment is endoscopically defined as the proximal limit of rugal folds. Biopsies taken at the proximal limit of rugal folds almost always show intestinal, cardiac, and/or oxyntocardiac epithelia. They are almost never composed of gastric oxyntic epithelium [43]. The squamo-oxyntic gap extends to a variable length distal to the end of the tubular esophagus and the proximal limit of rugal folds [13].

In the patients who have vCLO, therefore, the squamo-oxyntic gap consists of two components (Fig. 4.6):

(a) The part of the gap above the proximal limit of rugal folds in the tubular esophagus, which is equivalent to the endoscopically visible CLO.
(b) The part of the gap distal to the proximal limit of rugal folds in the saccular structure distal to the tubular esophagus. This part will be regarded at endoscopy as proximal stomach ("gastric cardia") because it is distal to the proximal limit of rugal folds, which is the endoscopic definition of the GOJ.

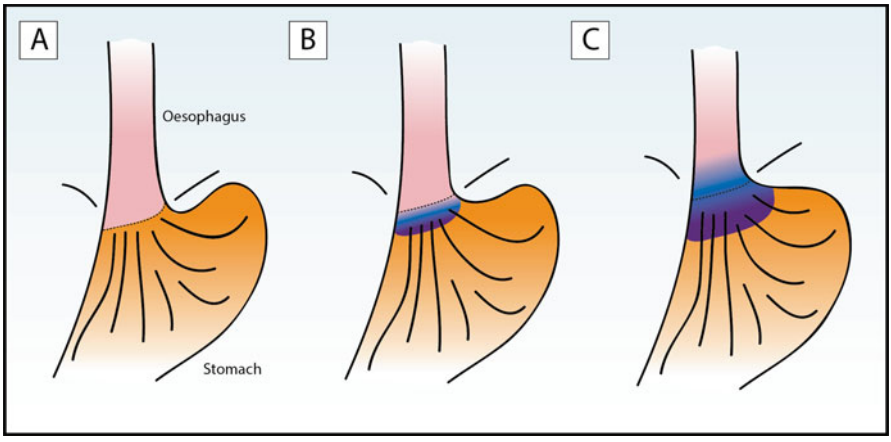

Fig. 4.6 Progression of the squamo-oxyntic gap. (**a**) Normal state with no squamo-oxyntic gap. Dashed line is the anatomic and endoscopic GOJ. (**b**) Squamo-oxyntic gap limited to the dilated distal esophagus. This is presently called proximal stomach (gastric cardia) because it is distal to the end of the tubular esophagus and the proximal limit of rugal folds. The endoscopic GOJ (*dashed line*) has moved proximally. (**c**) Final phase of progression where LOS damage has led to sufficient reflux into the esophageal body to cause vCLO. Note: As LOS damage progresses, the tubular esophagus shortens and the angle of His become more obtuse. The dilated distal esophagus functionally becomes part of the gastric reservoir and develops rugal folds

The squamo-oxyntic gap is, in reality, the full histologic extent of the columnar lined esophagus. It is always longer that the endoscopic vCLO [13, 28]. Recognition that its distal part, which is distal to the tube and in mucosa that has rugal folds, is esophageal rather than gastric permits accurate definition of the full extent of the CLO [13]. The full extent of the CLO cannot be measured endoscopically because the point of transition of metaplastic oxyntocardiac epithelium to gastric oxyntic epithelium (the true GOJ) cannot be seen. It must be measured by multilevel biopsies that measure the squamo-oxyntic gap distal to the endoscopic GOJ [43].

The Distal Part of the Squamo-Oxyntic Gap Correlates with Damage to the Abdominal Los

There is evidence that the presence of a squamo-oxyntic gap is associated with shortening of the abdominal LOS, beginning distally and extending proximally at a rate of progression that varies in different people. Robertson et al. [15], in their study of asymptomatic volunteers with and without central obesity reported findings in high resolution pH metry (using 12 sensors) and manometry (36 sensors). There was no evidence of excessive reflux 5 cm above the LOS in either group. The persons with central obesity had shortening of the LOS, attributable to loss of the distal (abdominal) component. Derakhshan et al. reporting the histopathological characteristics of the cardiac epithelium in Robertson et al. asymptomatic volunteers showed that cardiac epithelium had similar intensity of inflammation and immunohistochemical features to vCLO without intestinal metaplasia. They concluded that expansion of cardiac epithelium in healthy volunteers resulted from columnar metaplasia of the squamous epithelium of the distal esophagus and that this was greater in persons with central obesity than those without. Their data essentially proves that even the small squamo-oxyntic gaps seen in this region are similar to nonintestinalized columnar metaplasia in vCLO. Other studies have shown a marked difference in the amount of inflammation in the mucosa between cardiac epithelium at the junction and immediately adjacent gastric oxyntic epithelium [45]. Oberg et al. [44] showed that patients who had a squamo-oxyntic gap in a retroflex endoscopic biopsy taken from within 10 mm from a normal appearing squamocolumnar junction had a greater likelihood of having a shortened abdominal LOS compared to patients who did not have cardiac and/or oxyntocardiac epithelium.

Definition of the Gastroesophageal Junction (GOJ)

The evidence presented provides strong evidence that the squamo-oxyntic gap, however small its extent, is associated with evidence of GORD and an abnormal LOS. It can therefore not be part of the stomach. The end of the esophagus must be

the distal limit of the squamo-oxyntic gap or the point where the squamo-oxyntic gap meets the proximal limit of gastric oxyntic epithelium. This is the true GOJ. It is only concordant with the endoscopic definitions of the GOJ (end of the tubular esophagus and proximal limit of rugal folds) only in normal patients with a zero squamo-oxyntic gap. This is extremely uncommon. GORD is an extremely common human disease in the USA, like coronary atherosclerosis.

The proof that the true GOJ is the junction between the distal limit of the squamo-oxyntic gap and the proximal limit of gastric oxyntic epithelium is the presence of esophageal submucosal glands underlying metaplastic columnar epithelia. Chandrasoma et al. [13] reported the findings of ten esophagectomy specimens that had a sharp transition from the tube to the sac at the exact location of well-defined proximal rugal folds. Eight patients had adenocarcinoma of the esophagus secondary to Barrett esophagus and 2 had squamous carcinoma without visible columnar metaplasia in the tubular esophagus. In the 8 patients with adenocarcinoma, the squamo-oxyntic gap extended 10.3–20.5 mm distal to the end of the tubular esophagus (Fig. 4.7a). In the 8 patients with adenocarcinoma who had columnar metaplasia in the tubular esophagus, the entire squamo-oxyntic gap consisted of the visible part in the tubular esophagus (vCLO) plus the 10.3–20.5 cm that was in the sac distal to the end of the tubular esophagus and proximal limit of rugal folds (Fig. 4.7a). In the two patients with squamous carcinoma without any columnar metaplasia in the tubular esophagus, the total squamo-oxyntic gap was 3.1 and 4.3 mm and entirely in the pouch distal to the end of the tubular esophagus. Squamous carcinoma has no association with GORD; these gaps are similar to those seen in asymptomatic people at autopsy. In all patients, the distribution of columnar epithelia was indicative of a single gap with intestinal metaplasia in the proximal region adjacent to the squamocolumnar junction and oxyntocardiac epithelium distally adjacent to gastric oxyntic epithelium [34] (Fig. 4.7a).

One of the advantages of the resected specimens used in this study is that it permits examination of the full thickness of the esophagus. In the ten specimens studied [13], we mapped the submucosal glands and compared their distribution to the overlying epithelia (Fig. 4.7b, c). Submucosal glands were concordant within 5 mm of the squamo-oxyntic gap, i.e. they extended to the gap or within 5 mm proximal to the gap, irrespective of the length of the gap. Submucosal glands were never seen under gastric oxyntic epithelium.

Submucosal glands develop in the fetal esophagus only after squamous epithelium has replaced fetal esophageal columnar epithelia [46]. As such, it is limited to the esophagus, never being seen in the normal stomach. The finding that the squamo-oxyntic gap and submucosal glands are concordant in length essentially proves that the sac distal to the end of the tubular esophagus is a dilated, GORD-damaged, distal esophagus to the extent that it is lined by the squamo-oxyntic gap.

Sarbia et al. [28], in a similar study of esophagectomy specimens in 36 patients with squamous carcinoma where all patients had the entire tubular esophagus lined by squamous epithelium, i.e. there was no vCLO. They sectioned the entire area distal to the squamocolumnar junction and measured the epithelial types. Cardiac epithelium was present along the entire circumference of the squamocolumnar

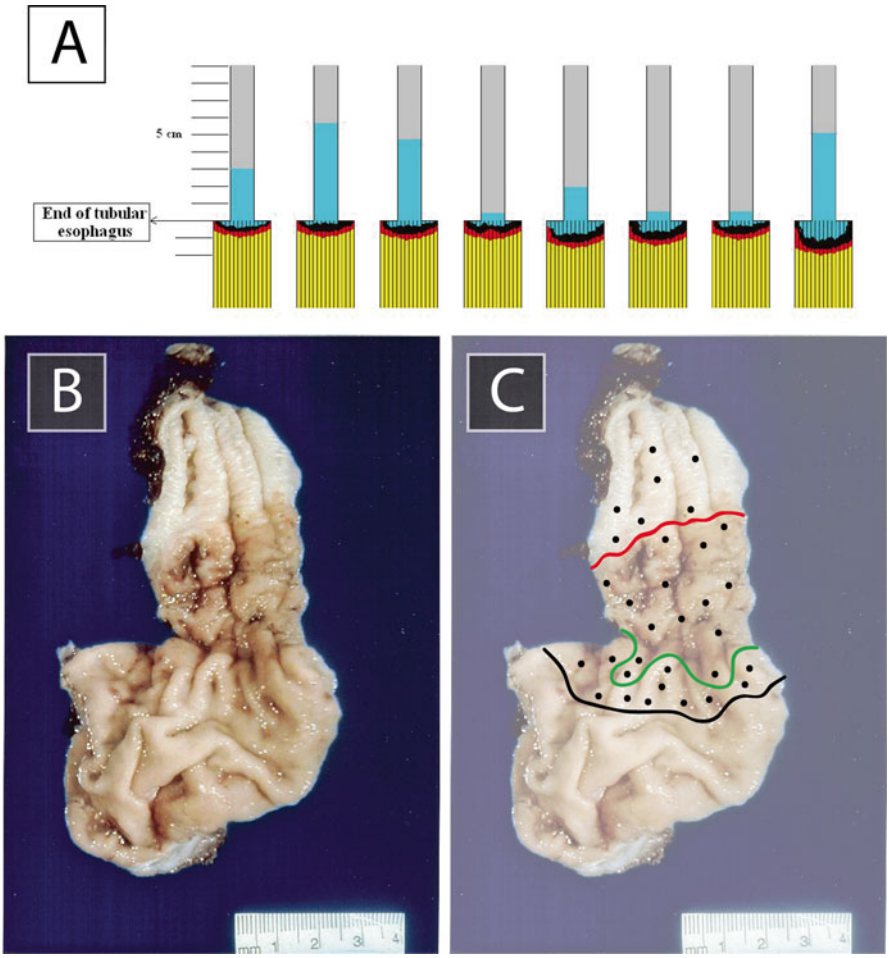

Fig. 4.7 (**a**) Esophagectomy specimens in 8 patients with adenocarcinoma arising in Barrett esophagus. The squamo-oxyntic gap extends into the dilated distal esophagus for 10.3–20.5 mm and is associated with a variable segment of vCLO in the tubular esophagus. *Blue* = intestinal metaplasia; *red* = cardiac epithelium; *black* = oxyntocardiac epithelium; *yellow* = gastric oxyntic epithelium; *black vertical lines* indicate rugal folds. (**b**, **c**) Esophagectomy specimen of patient at right end of (**a**) showing 5.5 cm of vCLO and 2.0 cm of dilated distal esophagus (LOS shortening). *Black dots* = submucosal glands; *red line* = squamocolumnar junction; *green*; distal limit of intestinal metaplasia; *black line* = proximal limit of gastric oxyntic mucosa (distal limit of squamo-oxyntic gap/true GOJ

junction in 20/36 patients, part of the circumference in 15 and completely absent in 1. The combined length of cardiac and oxyntocardiac epithelium in the squamo-oxyntic gap was a minimum of 1–12 mm (median 4 mm) and the maximum length was between 5 and 28 mm (median 11 mm). In 8 (25 %) patients, cardiac and/or oxyntocardiac epithelium was situated over submucosal glands and in a ninth case

these epithelia were found overlying squamous lined ducts of these submucosal glands. This study showed that all patients with squamous carcinoma had a dilated distal esophagus composed of cardiac and oxyntocardiac epithelium separating the squamous epithelium from gastric oxyntic epithelium. The low (25 %) percentage of submucosal glands in this study is likely due to the fact that, in this non-GORD population, the median maximum length of the gap was 11 mm. Submucosal glands were never seen underlying gastric oxyntic epithelium.

The true GOJ cannot be seen at endoscopy because the dilated distal esophagus and proximal stomach both have rugal folds. With standard endoscopy, it is not possible to differentiate oxyntocardiac and gastric oxyntic epithelium. It is possible that newer endoscopic modalities, such as optical coherence tomography, can do this. At present, though, only histologic examination is capable of identifying the true GOJ.

Genesis and Progression of the Squamo-Oxyntic Gap

We can now explain the genesis of the squamo-oxyntic gap and how it relates to GORD. The gap *always* results from columnar metaplasia of the esophageal squamous epithelium by its exposure to gastric juice. Exposure of esophageal squamous epithelium to gastric juice occurs in two distinct ways and by two completely different mechanisms, clearly separated in time with some overlapping in the middle.

Columnar Metaplasia Due to LOS Effacement Without Reflux

In a patient with a normal LOS at the onset of disease, usually in early life, the LOS is placed under pressure from below during episodic over-eating [14, 15]. The effacement of 5–7 mm of the distal LOS that occurs during a heavy meal causes a proximal migration of the pH transition point within the LOS causing the distal 5–7 mm of esophageal squamous epithelium to be exposed to the acid pocket in the stomach [15]. This causes squamous epithelium injury, which is reversible. Over the years, however, repeated damage of the squamous epithelium ultimately results in irreversible columnar metaplasia. This happens very slowly, one cell at a time, usually 1–5 mm per decade with rare outliers. Those with slower rates of columnar metaplasia are closer to a zero squamo-oxyntic gap. Those with >5 mm/decade of columnar metaplasia progress to severe LOS damage early in life.

Replacement of the squamous epithelium with columnar (cardiac) epithelium results in loss of LOS function by a mechanism that is unknown. This results in LOS shortening, beginning at the distal end and progressing cephalad as the squamo-oxyntic gap increases in length. We have suggested that the loss of loss of LOS pressure when columnar metaplasia occurs results from disruption of nerve endings in the squamous epithelium that may be the afferent arc of an intramural reflex that maintains tonic contraction of LOS muscle.

In patients who do not develop GORD, the LOS shortening and the squamo-oxyntic gap never exceeds 10 mm in their lifetime. They have lived within the reserve capacity of their LOS. This is analogous to a person with <60 % narrowing of coronary arteries who do not develop ischemic heart disease. With an original abdominal LOS length of 25–30 mm, 10 mm shortening means there is a residual abdominal LOS length of 15–20 mm. Even with the shortening of 5–7 mm during a heavy meal, sphincter failure and reflux is limited if it ever occurs [15, 17] (Table 4.1). It is only in the patient with no GORD and no LOS damage that the GOJ is at the junction of the tube and sac and at the proximal limit of rugal folds. Such patients with a zero squamo-oxyntic gap are rare, analogous to adults with absolutely no coronary atherosclerosis that is also rare. Almost all people in the population have a dilated distal esophagus. This is typically <5 mm in people without GORD (autopsy [27, 29] and asymptomatic volunteers [15]) and increases in length as the severity of GORD increases [13].

Columnar Metaplasia of the Esophageal Body Due to LOS Failure and Reflux

As the squamo-oxyntic gap and abdominal LOS shortening increases >10 mm, sphincter failure begins to occur, usually in the postprandial period where additional LOS effacement occurs with a heavy meal [13]. The pH test becomes abnormal, symptoms commonly begin, and the patient requires medical therapy, at least in the postprandial phase.

At this onset of symptoms of GORD, the patient has reached a critical stage in the progression of the disease. Even a slight additional decrease in LOS length results in an exponential increase in the likelihood of LOS failure. Exposure of the squamous epithelium of the body of the esophagus, all the way to the arch of the aorta, which is the nadir of intraluminal esophageal pressure [10], increases rapidly. Increasing erosive esophagitis and vCLO occur in an unpredictable manner at this stage in the individual patient. There are many unknown factors such as the volume of reflux, pH of refluxate, and innate resistance of the squamous epithelium to acid exposure, that determine who develops erosions and who develops vCLO. LOS shortening >15 mm indicates high risk of sphincter failure and reflux to an extent that the patient is likely to develop severe symptoms independent of meals with symptom control needing dose escalation and likely to be incomplete. They are more likely to have erosive esophagitis and vCLO. Ideally, such patients should have had an LOS assessment before this stage is reached and a repair already done to prevent this.

This way of looking at GORD shows that a person can destroy 33–40 % of the abdominal LOS (i.e., 10 mm of a 25–30 mm sphincter) and still have a competent sphincter. These patients do not have significant sphincter failure and the body of the esophagus is spared (Table 4.1). There are no symptoms that result from reflux of gastric juice into the esophageal body. The only evidence of this early GORD is

the histologic squamo-oxyntic gap in the dilated distal esophagus. This is misunderstood by present endoscopic and pathologic diagnostic criteria as "normal proximal stomach (gastric cardia)" and completely ignored [12].

The occurrence of the first symptom of postprandial heartburn indicates LOS shortening that is >33–40 % of its original length. It does not signify early disease. It is already associated with a considerable amount of LOS damage. Symptomatic GORD is a late manifestation of GORD in terms of severity of LOS damage. If endoscopy is performed relatively soon after the onset of GORD symptoms, 5–10 % of patients already will have vCLO and has entered the phase of irreversibility. In the Pro-GERD study [4], 240/2721 (8.8 %) of patients had vCLO. These patients are at risk for progression to adenocarcinoma without any reliable method of prevention. The present problem is that endoscopy is not indicated even in these patients. Therefore, vCLO will remain undetected in the 8.8 % of patients in the Pro-GERD study without the special study-driven endoscopy [4].

In the Pro-GERD study, continued empiric treatment with acid suppressive drugs resulted in the conversion of another 241/2481 (9.7 %) patients from no vCLO to vCLO within 5 years [4]. The vCLO lengths in these patients ranged from 1 to >5 cm. This was not the slow increase in the length of columnar metaplasia that was caused by LOS damage. It was the result of sufficient LOS damage to cause reflux into the esophageal body. The LOS damage had reached critical mass. Every millimeter of further LOS damage increased the number of events of sphincter failure and episodes of reflux into the body of the esophagus. It is as if the disease exploded from being limited to the LOS to involve the body of the esophagus *en masse*. This resulted in a rapid development of columnar metaplasia in the body of the esophagus adding the second dimension to the dilated distal esophagus, which was the vCLO.

All patients who have vCLO will have a dilated distal esophagus, commonly >10 mm long (Fig. 4.7). In Chandrasoma et al. [13], eight patients with vCLO had a dilated distal esophagus measuring 10.3–20.5 mm. The length of vCLO in the tubular esophagus varied from 0.5 to 5.5 cm. Patients who do not have vCLO, usually have a dilated distal esophagus that is <10 mm. The two patients with squamous carcinoma had no vCLO and a dilated distal esophagus of 3.1 and 4.6 mm.

Unfortunately, symptom severity or duration does not correlate perfectly with LOS damage. A minority of patients with vCLO, who almost always have severe LOS damage, may reach that state without significant symptoms. These people with asymptomatic vCLO will remain undetected unless screening for vCLO becomes feasible, which is unlikely.

A New Pathologic Test of Los Damage

If the squamo-oxyntic gap and the dilated distal esophagus are concordant in length in patients whose gap has not extended up into the tubular esophagus, it must follow that the former is an accurate measure of the latter. We now have a histologic method

of measuring the length of the dilated distal esophagus, which is equal to shortening of the abdominal segment of the LOS. Like most histologic measurements, it is highly accurate if the appropriate specimen is available.

Measurement at Autopsy

We highly recommend that a routine examination of the entire circumference of the squamocolumnar junction including 25 mm distal to this be added to autopsy examinations in academic centers that have an interest in GORD. If the peritoneal reflection can be identified, the vertical section from the squamocolumnar junction should ideally extend to this point. Most of these specimens will have severe autolysis that precludes accurate definition, but there will be some with adequate histology to measure the squamo-oxyntic gap. In procuring tissue from organ donors, routine harvesting of this region, if done, will provide potentially invaluable data. Direct measurement of the gap between the distal limit of squamous epithelium and proximal limit of gastric oxyntic epithelium using an ocular micrometer will provide an exact measurement of the gap. Use of a D-PAS stain will help distinguish oxyntocardiac from gastric oxyntic epithelium, increasing accuracy of measurement.

Measurement in Esophago-Gastrectomy Specimens

Every esophagogastrectomy specimen that has the junctional region should be carefully evaluated for landmarks such as the squamocolumnar junction, point of flaring of the tubular esophagus, peritoneal reflection, and the proximal limit of rugal folds. In patients who have no vCLO in the tubular esophagus, the entire circumference of the 25 mm distal to the squamocolumnar junction should be sectioned vertically to measure the squamo-oxyntic gap. In patients who have a vCLO, the entire vCLO segment plus the 25 mm distal to the end of the tubular esophagus should be examined. These specimens are not uncommon in large academic centers. We and Sarbia et al. [13, 28] showed the ease with which these studies can be done and the accuracy of the measurement of the squamo-oxyntic gap. These specimens also permit assessment of the correlation between submucosal glands and surface epithelial type. The lack of interest in such studies is incredible.

Measurement at Endoscopy with Present Biopsy Forceps

We suggest that routine biopsies be taken at every upper GI endoscopy with the intent of measuring the squamo-oxyntic gap in academic centers that have an interest in GORD. In general, the presence of vCLO at endoscopy means that there is

severe LOS damage and the need to measure the squamo-oxyntic gap has only academic value to confirm the relationship between vCLO and the length of the squamo-oxyntic gap. For this purpose, measured biopsies should be taken from the proximal limit of rugal folds for a vertical distance of 25 mm distally.

The measurement of the squamo-oxyntic gap is most valuable in patients who are endoscopically normal. In these patients measuring the gap routinely will provide the baseline database that will differentiate all people in the population in terms of the length of the squamo-oxyntic gap (= dilated distal esophagus). There is presently excellent data that shows that patients without GORD symptoms (at autopsy [27, 29] and asymptomatic volunteers [15]) have very short (<5 mm) squamo-oxyntic gaps. As GORD symptoms develop, the length of the gap increases.

Measuring the gap routinely will provide a highly precise basis for correlating LOS damage with symptom severity, failure of medical therapy, erosive esophagitis, vCLO, vCLO with intestinal metaplasia and neoplasia. Such data will be a platform for making decisions on how best to manage GORD patients in the future. Without adequate study, the data will never be accumulated and whether measurement of the squamo-oxyntic gap is a useful test for GORD will never be known. It is not as if GORD was a disease that is easily diagnosed by any available test at present. It is not as if GORD is a disease whose treatment is highly effective. The academic community should be desperately seeking new methods of studying the disease. They are not.

The biopsy technique in the patient without vCLO with present equipment is as follows: the area defined proximally by the squamocolumnar junction and distally by a point 25 mm distally is divided into the following five segments: (a) squamocolumnar junction to 5 mm, (b) 5–10 mm, (c) 10–15 mm, (d) 15–20 mm, (e) 20–25 mm. It is likely that the only biopsies necessary for practical assessment of the squamo-oxyntic gap are the first three. This will provide three biopsies:

Specimen A: biopsy (2–4 quadrant) straddling the squamocolumnar junction, aiming to sample the area zero to 5 mm distal to the junction;
Specimen B: biopsy immediately distal to specimen A, aiming to sample the area 5–10 mm from the junction;
Specimen C: biopsy immediately distal to specimen B, aiming to sample the area 10–15 mm from the junction.

Given that the open biopsy forceps measures 4 mm, a reasonably accurate sampling of this region can be performed. The biopsy protocol will permit classification of patients into the following grades of shortening of the abdominal segment of the LOS (Table 4.3). Adding another biopsy that samples the area 15–20 mm from the squamocolumnar junction will permit classification of LOS shortening into two further grades: 15–20 mm and >20 mm. However, since the objective is to prevent patients from reaching the critical 15 mm of shortening, this is probably not necessary. Waiting for >20 mm shortening is equivalent to waiting for the patient to develop uncontrollable reflux and vCLO, which is our definition of failed treatment. It is the present point in the GORD treatment algorithm that GORD is taken seri-

Table 4.3 Interpretation of the results of the recommended biopsy protocol

Biopsy A	Biopsy B	Biopsy C	LOS(a) shortening	Likelihood of reflux
Sq + GOM	GOM	GOM	Zero	Zero
Sq + MCM + GOM	GOM	GOM	<5 mm	Near zero
Sq + MCM	MCM + GOM	GOM	5–10 mm	Mild
Sq + MCM	MCM	MCM + GOM	10–15 mm	Moderate
Sq + MCM	MCM	MCM	>15 mm	Severe

Sq squamous epithelium, *MCM* metaplastic columnar epithelium, consisting of oxyntocardiac epithelium +/− cardiac epithelium +/− intestinal epithelium, *GOM* gastric oxyntic epithelium
Compare with Table 4.1

ously as a disease and the point at which endoscopy is indicated. Endoscopy must happen much earlier if early diagnosis by our method is to be effective.

Initially, there should be a phase of assessment (like a phase 1 trial), where these biopsies should be limited to academic centers with an interest in GORD and where data relating to symptoms, pH, and impedance studies, and manometry are carefully documented. This will permit correlation of the biopsy findings with clinical and objective measurement of reflux into the esophagus. Without these studies, the measurement of the gap will have little meaning. The correlation between LOS shortening and severity of reflux will not be exact. Sphincter failure depends on the residual functional LOS length rather than LOS shortening and this in turn depends on the original LOS length, which varies in different people. When the squamo-oxyntic gap has been studied and correlations established, the biopsy requirement will move into the mainstream if the data shows that the assessment is valuable for clinical decisions. It is also important to follow these test patients. Ultimately, the test will have greatest value if the test results permit prediction of future progression. This will allow intervention in a subgroup of patients with GORD before they reach the critical point of 15 mm shortening.

There is evidence that this biopsy protocol separates GORD patients into 3 groups. Ringhofer et al. [43], using a biopsy protocol that extended from the proximal limit of rugal folds (endoscopic GOJ) to 10 mm distally, showed that the squamo-oxyntic gap extended to at least 5 mm distal to the end of the tubular esophagus in 81 % (i.e., 19 % had a gap <5 mm) and to at least 10 mm in 28 % of patients (i.e., 53 % had a gap of 5–10 mm and 28 % had a gap >10 mm). There is a strong probability that these three groups will have different severity of GORD.

There is one asymptomatic person who has an accurately measured squamo-oxyntic gap. One of the authors (PC) had his gastroenterologist add an upper endoscopy at the time of his screening colonoscopy with instructions to biopsy distal to the squamocolumnar junction at 5 mm intervals for 20 mm. Endoscopy was normal. The biopsy taken from 0 to 5 mm from the squamocolumnar junction consisted of one focus of cardiac epithelium measuring 0.2 mm (two foveolar complexes), oxyntocardiac epithelium which predominated and gastric oxyntic epithelium. The three biopsies distal to 5 mm all consisted of normal gastric oxyntic epithelium only. This led to the conclusion that the squamo-oxyntic gap was 4 mm with a mixture of

oxyntocardiac epithelium (dominant) and cardiac epithelium. There was no intestinal metaplasia. This correlates with the absence of GORD symptoms and a normal esophageal body at endoscopy, similar to autopsy population without symptoms during life [27, 29] and asymptomatic volunteers [15].

Future Ideal Endoscopic Measurement

If studies show that the measurement of the squamo-oxyntic gap in the dilated distal esophagus (i.e., the length of shortening of the abdominal LOS) has value, it is likely that more accurate methods of measuring the squamo-oxyntic gap will be developed. The two possible methods of measurement are as follows: (a) the development of a new biopsy forceps that can take a vertical biopsy with its proximal end at the squamocolumnar junction and measuring 15–20 mm. This will permit a precise measurement of the squamo-oxyntic gap. (b) Optical coherence tomography that can be used at endoscopy. This potentially has the technical capability of distinguishing between the thinner metaplastic epithelia with inflammation and disordered glands in the squamo-oxyntic gap from the thicker, less inflamed, more orderly glandular structure of normal gastric oxyntic epithelium. This would, if successful, provide a measurement of the squamo-oxyntic gap with micrometric precision.

The technology to measure the squamo-oxyntic gap is available and can be easily improved to increase accuracy. It is only the will to perform the measurement that is missing. We suggest that this failure is the result of misunderstanding of normal anatomy and histology and the blind following of an incorrect definition of the GOJ that has no evidence in support of it and much evidence to refute it.

Conclusion

We will not progress in the understanding, diagnosis, and treatment of this disease as long as we continue to define GORD by symptoms and complications, which represent a relatively late stage of LOS damage [19]. It is equivalent to defining ischemic heart disease by angina. Like fatal myocardial infarction was the first clinical sign of ischemic heart disease in some patients, the presence of esophageal adenocarcinoma is the first sign of clinical GORD in some patients.

The path to early diagnosis that will pave the way to identifying high-risk patients is by measuring LOS damage by its concordance with the histologic squamo-oxyntic gap in the dilated distal esophagus [12, 22].

We must recognize that the dilated distal esophagus is not the normal proximal stomach; it is the early pathology of GORD that remains unrecognized by present definitions and diagnostic criteria.

References

1. Kahrilas PJ, Shaheen NJ, Vaezi MF. American gastroenterological association medical position statement on the management of gastroesophageal reflux disease. Gastroenterology. 2008;135:1383–91.
2. Spechler SJ, Zeroogian JM, Antonioli DA, Wang HH, Goyal RK. Prevalence of metaplasia at the gastroesophageal junction. Lancet. 1994;344:1533–6.
3. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association medical position statement on the management of Barrett's esophagus. Gastroenterology. 2011;140:1084–91.
4. Malfertheiner P, Nocon M, Vieth M, Stolte M, Jasperson D, Keolz HR, Labenz J, Leodolter A, Lind T, Richter K, Willich SN. Evolution of gastro-oesophageal reflux disease over 5 years under routine medical care—the ProGERD study. Aliment Pharmacol Ther. 2012;35:154–64.
5. Pohl H, Sirovich B, Welch HG. Esophageal adenocarcinoma incidence: are we reaching the peak? Cancer Epidemiol Biomarkers Prev. 2010;19:1468–70.
6. Allison PR. Peptic ulcer of the oesophagus. Thorax. 1948;3:20–42.
7. Morson BC, Belcher BR. Adenocarcinoma of the oesophagus and ectopic gastric mucosa. Br J Cancer. 1952;6:127–30.
8. Leodolter A, Nocon M, Vieth M, Lind T, Jasperson D, Richter K, Willich S, Stolte M, Malfertheiner P, Labenz J. Progression of specialized intestinal metaplasia at the cardia to macroscopically evident Barrett's esophagus: an entity of concern in the Pro-GERD study. Scand J Gastroenterol. 2012;47:1429–35.
9. Bonavina L, Evander A, DeMeester TR, et al. Length of the distal esophageal sphincter and competency of the cardia. Am J Surg. 1986;151:25–34.
10. Zaninotto G, DeMeester TR, Schwizer W, Johansson KE, Cheng SC. The lower esophageal sphincter in health and disease. Am J Surg. 1988;155:104–11.
11. Stein HJ, Liebermann-Meffert D, DeMeester TR, Siewert JR. Three-dimensional pressure image and muscular structure of the human lower esophageal sphincter. Surgery. 1995;117:692–8.
12. Chandrasoma P, Wijetunge S, Ma Y, DeMeester S, Hagen J, DeMeester T. The dilated distal esophagus: a new entity that is the pathologic basis of early gastroesophageal reflux disease. Am J Surg Pathol. 2011;35:1873–81.
13. Chandrasoma P, Makarewicz K, Wickramasinghe K, Ma YL, DeMeester TR. A proposal for a new validated histologic definition of the gastroesophageal junction. Human Pathol. 2006;37:40–7.
14. Ayazi S, Tamhankar A, DeMeester SR, et al. The impact of gastric distension on the lower esophageal sphincter and its exposure to acid gastric juice. Ann Surg. 2010;252:57–62.
15. Robertson EV, Derakhshan MH, Wirz AA, Lee YY, Seenan JP, Ballantyne SA, Hanvey SL, Kelman AW, Going JJ, McColl KE. Central obesity in asymptomatic volunteers is associated with increased intrasphincteric acid reflux and lengthening of the cardiac mucosa. Gastroenterology. 2013;145:730–9.
16. Clarke AT, Wirz AA, Manning JJ, Ballantyne SA, Alcorn DJ, McColl KE. Severe reflux disease is associated with an enlarged unbuffered proximal gastric acid pocket. Gut. 2008;57:292–7.
17. Kahrilas PJ, Shi G, Manka M, Joehl RJ. Increased frequency of transient lower esophageal sphincter relaxation induced by gastric distension in reflux patients with hiatal hernia. Gastroenterology. 2000;118:688–95.
18. Rodrigo J, Hernandez CJ, Vidal MA, Pedrosa JA. Vegetative innervation of the esophagus. III. Intraepithelial endings. Acta Anat. 1975;92:242–58.
19. Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones B, The Global Consensus Group. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol. 2006;101:1900–20.

20. McClave SA, Boyce Jr HW, Gottfried MR. Early diagnosis of columnar lined esophagus: a new endoscopic diagnostic criterion. Gastrointest Endosc. 1987;33:413–6.
21. Sharma P, McQuaid K, Dent J, Fennerty B, Sampliner R, Spechler S, Cameron A, Corley D, Falk G, Goldblum J, Hunter J, Jankowski J, Lundell L, Reid B, Shaheen N, Sonnenberg A, Wang K, Weinstein W. A critical review of the diagnosis and management of Barrett's esophagus: the AGA Chicago Workshop. Gastroenterology. 2004;127:310–30.
22. Chandrasoma PT. Histologic definition of gastro-esophageal reflux disease. Curr Opin Gastroenterol. 2013;29:460–7.
23. Paull A, Trier JS, Dalton MD, Camp RC, Loeb P, Goyal RK. The histologic spectrum of Barrett's esophagus. N Eng J Med. 1976;295:476–80.
24. Chandrasoma P. Controversies of the cardiac mucosa and Barrett's esophagus. Histopathology. 2005;46:361–73.
25. Chandrasoma P. Pathophysiology of Barrett's esophagus. Semin Thoracic Cardiovasc Surg. 1997;9:270–8.
26. Chandrasoma PT, Wijetunge S, DeMeester SR, Hagen JA, DeMeester TR. The histologic squamo-oxyntic gap: an accurate and reproducible diagnostic marker of gastroesophageal reflux disease. Am J Surg Pathol. 2010;34:1574–81.
27. Chandrasoma PT, Der R, Ma Y, et al. Histology of the gastroesophageal junction: an autopsy study. Am J Surg Pathol. 2000;24:402–9.
28. Sarbia M, Donner A, Gabbert HE. Histopathology of the gastroesophageal junction. A study on 36 operation specimens. Am J Surg Pathol. 2002;26:1207–12.
29. Kilgore SP, Ormsby AH, Gramlich TL, et al. The gastric cardia: fact or fiction? Am J Gastroenterol. 2000;95:921–4.
30. Park YS, Park HJ, Kang GH, Kim CJ, Chi JG. Histology of gastroesophageal junction in fetal and pediatric autopsy. Arch Pathol Lab Med. 2003;127:451–5.
31. Glickman JN, Fox V, Antonioli DA, Wang HH, Odze RD. Morphology of the cardia and significance of carditis in pediatric patients. Am J Surg Pathol. 2002;26:1032–9.
32. Chandrasoma PT, Lokuhetty DM, DeMeester TR, et al. Definition of histopathologic changes in gastroesophageal reflux disease. Am J Surg Pathol. 2000;24:344–51.
33. Odze RD. Unraveling the mystery of the gastroesophageal junction: a pathologist's perspective. Am J Gastroenterol. 2005;100:1853–67.
34. Chandrasoma PT, Der R, Ma Y, Peters J, DeMeester T. Histologic classification of patients based on mapping biopsies of the gastroesophageal junction. Am J Surg Pathol. 2003;27:929–36.
35. Chandrasoma PT, Der R, Dalton P, Kobayashi G, Ma Y, Peters J, DeMeester T. Distribution and significance of epithelial types in columnar lined esophagus. Am J Surg Pathol. 2001;25:1188–93.
36. Katz PO, Castell DO, Chen Y, Andersson T, Sostek MB. Intragastric acid suppression and pharmacokinetics of twice-daily esomeprazole: a randomized, three-way crossover study. Aliment Pharmacol Ther. 2004;20:399–406.
37. Allison PR, Johnstone AS. The oesophagus lined with gastric mucous membrane. Thorax. 1953;8:87–101.
38. Barrett NR. Chronic peptic ulcer of the oesophagus and "oesophagitis". Br J Surg. 1950;38:175–82.
39. Barrett NR. The lower esophagus lined by columnar epithelium. Surgery. 1957;41:881–94.
40. Hayward J. The lower end of the oesophagus. Thorax. 1961;16:36–41.
41. Jain R, Aquino D, Harford WV, Lee E, Spechler SJ. Cardiac epithelium is found infrequently in the gastric cardia. Gastroenterology. 1998;114:A160.
42. Derakhshan MH, Robertson EV, Lee YY, Harvey T, Ferrier RK, Wirz AA, Orange C, Ballantyne SA, Hanvey SL, Going JJ, McColl KE. In healty volunteers, immunohistochemistry supports squamous to columnar metaplasia as mechanism of expansion of cardia, aggravated by central obesity. Gut. 2015;64(11):1705–14.

43. Ringhofer C, Lenglinger J, Izay B, Kolarik K, Zacherl J, Fisler M, Wrba F, Chandrasoma PT, Cosentini EP, Prager G, Riegler M. Histopathology of the endoscopic esophagogastric junction in patients with gastroesophageal reflux disease. Wien Klin Wochenschr. 2008;120:350–9.
44. Oberg S, Peters JH, DeMeester TR, et al. Inflammation and specialized intestinal metaplasia of cardiac mucosa is a manifestation of gastroesophageal reflux disease. Ann Surg. 1997;226:522–32.
45. Lembo T, Ippoliti AF, Ramers C, Weinstein WM. Inflammation of the gastroesophageal junction (carditis) in patients with symptomatic gastroesophageal reflux disease; a prospective study. Gut. 1999;45:484–8.
46. Johns BAE. Developmental changes in the oesophageal epithelium in man. J Anat. 1952;86:431–42.

Part II
Cancer Progression in the Distal Esophagus

Chapter 5
Diagnosis by Endoscopy and Advanced Imaging of Barrett's Neoplasia

Anne-Fré Swager, Wouter L. Curvers, and Jacques J. Bergman

Abbreviations

AFI	Autofluorescence imaging
BE	Barrett's esophagus
BLI	Blue laser imaging
CLE	Confocal laser endomicroscopy
EAC	Esophageal adenocarcinoma
ETMI	Endoscopic trimodal
FICE	Fujifilm intelligent chromo-endoscopy
HD	High-definition
HGD	High-grade dysplasia
IMC	Intramucosal carcinoma
LGD	Low-grade dysplasia
NBI	Narrow band imaging
OCT	Optical coherence tomography
OFDI	Optical frequency domain imaging
VLE	Volumetric laser endomicroscopy
WLE	White light endoscopy

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Introduction

The incidence of esophageal adenocarcinoma (EAC) in the western world has increased sixfold over the past three decades and has a dismal prognosis when detected at a symptomatic stage [1]. Adenocarcinoma develops through a precursor lesion called Barrett's esophagus (BE) in a sequence of gradually evolving, histologically recognizable steps: intestinal metaplasia, low-grade dysplasia (LGD), high-grade dysplasia (HGD), intramucosal carcinoma (IMC), and eventually invasive carcinoma. These intermediate grades of dysplasia offer a window of opportunity for curative therapy.

In the last decade, endoscopic therapy has been become the treatment of choice for early Barrett's neoplasia (i.e., HGD and IMC), with an excellent prognosis and safety profile compared to surgical resection [2]. A prerequisite for endoscopic therapy is adequate patient selection; only patients with HGD and IMC have a virtual absent risk of lymph node metastasis and are therefore amendable for endoscopic therapy [3].

In patients with known BE, regular surveillance endoscopy with random biopsies is recommended to detect early neoplastic lesions at a curable stage [4]. However, these lesions are often small, focally distributed, and endoscopically poorly visible (Fig. 5.1). Random four-quadrant biopsies may easily miss early lesions, since only about 5 % of the Barrett's segment is sampled [5]. Moreover, this process is laborious and many endoscopists do not adhere to the protocol [6].

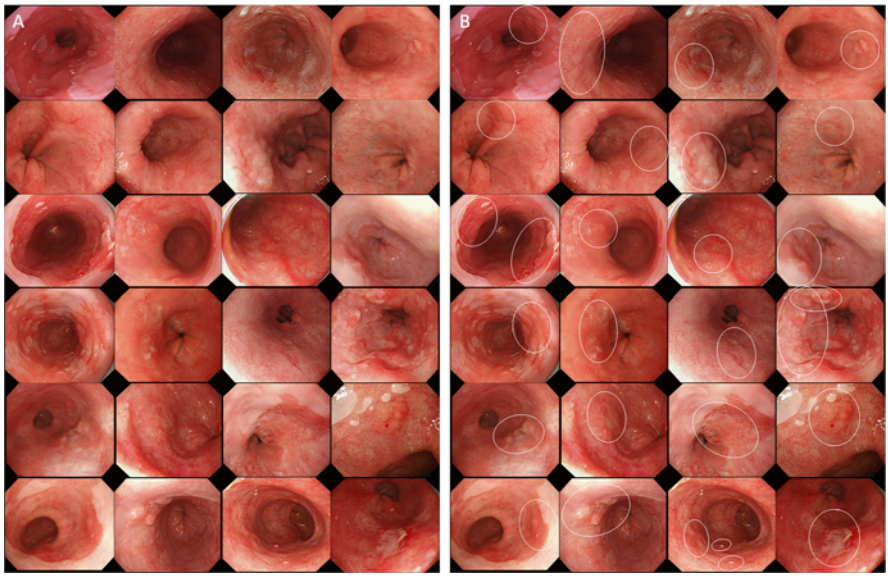

Fig. 5.1 Examples of subtle neoplastic lesions in Barrett's esophagus (**a**). The neoplastic lesions are encircled (**b**). Reproduced with permission from www.endosurgery.eu [75]

In recent years, many advanced imaging techniques have been developed to improve the detection of early Barrett's neoplasia.

In this chapter, we will discuss how to endoscopically diagnose early neoplasia during BE surveillance and how advanced imaging techniques may affect clinical management of BE either by improving the primary detection of early neoplastic lesions, allowing real-time diagnosis and decision making during endoscopy, or guiding the endoscopic workup and treatment. Parts of this review have been published earlier in specific publications on endoscopic workup of early Barrett's neoplasia and advanced imaging techniques by our group [7, 8]. The review was published in its entirety in Best Practice & Research Clinical Gastroenterology (published Online: December 03, 2014).

Endoscopic Diagnosis of Early Neoplasia in Barrett's Esophagus

The goal of endoscopic surveillance of patients with Barrett's esophagus is the detection of early neoplastic lesions. To ensure the detection of early neoplastic lesions there are three rules that should be followed. These rules relate to the endoscopic equipment used; the "detecting eye" of the endoscopist; and a systematic, meticulous approach.

Use Best Endoscope Available

High-resolution endoscopy using high-definition (HD) systems improve image resolution and reduce artifacts. The addition of magnification (zoom) endoscopy optically magnifies 150-fold without losing image quality, for optimally scrutinizing fine surface details [9, 10]. Most recent innovation enables the endoscopist to switch between two focus settings (dual focus, Evis Exera III 190, Olympus Inc., Tokyo, Japan): normal and near mode featuring close mucosal observation. Since early Barrett's esophagus neoplasia often presents as flat lesions with only subtle mucosal abnormalities, most experts agree that high-resolution endoscopy is the preferred method for the endoscopic evaluation of Barrett's esophagus.

You Do Not Detect What You See, You Detect What You Recognize

Up to 80 % of patients referred for workup of HGD/IMC without visible abnormalities will have at least one visible abnormality detected in their Barrett's esophagus upon endoscopic inspection by expert endoscopists [11, 12]. Although early BE

neoplasia generally presents as subtle flat lesions that may be difficult to detect, most state-of-the-art endoscopes do show these abnormalities to the experienced eye. Early neoplasia in BE is, however, relatively rare and most endoscopists do not encounter these lesions on a regular basis. The lack of familiarity of most endoscopists with the appearance of early gastrointestinal neoplasia thus becomes the limiting factor in the detection: "*You do not detect what you see, you detect what you recognize.*" Knowledge of the endoscopic appearance of early Barrett's neoplasia is thus essential for its diagnosis. Figure 5.1 shows a variety of subtle early neoplastic Barrett's esophagus lesions that may help endoscopists to recognize these lesions better in the future.

Perform a Systematic Endoscopic Inspection

The detection of gross mucosal abnormalities such as elevations, ulcerations, and nodularities in overview is fairly easy. For the detection of subtle abnormalities, a more careful and thorough inspection following a systematic approach is imperative. After intubation, the esophagus should be carefully cleaned out to remove any mucus or saliva. Then, it is important to remove all gastric secretions to prevent reflux into the esophagus that may interfere with inspection. Subsequently, the endoscope should be gradually withdrawn to examine the inflated Barrett's segment in overview for any mucosal irregularities and to describe the extent of the Barrett's esophagus according to the validated Prague C&M classification [13, 14]. After initial inspection, the inflated esophagus should be gradually deflated to reveal any irregularities that may have been stretched out during inflation (Fig. 5.2). Special attention should be paid to the area between 12 and 6 O'clock in the endoscopic view, since the majority of neoplastic lesions are located there [15]. Finally, it is important to inspect the transition of the Barrett's esophagus into the hiatal hernia in the retroflexed position, since abnormalities in this area are easily overlooked in the antegrade view (Fig. 5.2). All lesions detected during inspection should be classified according to the Paris Classification since the macroscopic appearance of these lesions is associated with the infiltration depth, which predicts the risk of submucosal invasion and thus the risk of lymph node involvement [16, 17]. Type 0-I and 0-IIc lesions carry a greater risk of submucosal invasion than do type 0-IIa, type 0-IIb, or combined types [15, 18]. Type 0-III lesions always have deep submucosal invasion and are accompanied by a dense fibrous reaction, and are therefore not suitable for endoscopic treatment.

Finally, biopsies should be obtained from each visible abnormality followed by random four-quadrant biopsies, always starting distally and working upward, so that the view is not obscured by bleeding. We follow the rule "look longer, biopsy less," since in our experience targeted biopsies performed after a thorough inspection contribute 80–90 % of the diagnosis of dysplasia [11, 12, 19]. At the present time, in the absence of visible abnormalities, random biopsies according to the Seattle protocol should still be performed [20].

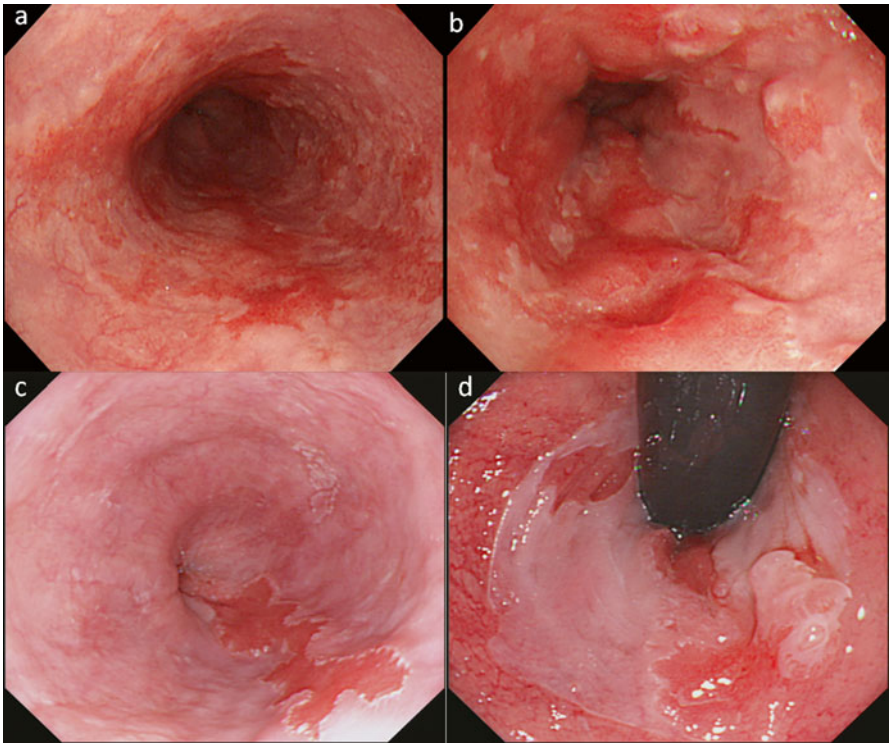

Fig. 5.2 Images of an early neoplastic lesion in Barrett's esophagus that is difficult to appreciate when the esophagus is fully inflated. By alternating inflation and suction, the lesion becomes more apparent (**a, b**). By looking in retroflex, lesions at the distal esophagus may be detected that would have been missed when only antegrade inspection would have been performed (**c, d**)

Advanced Imaging Techniques in Barrett's Esophagus

Detection of Early Neoplasia

For primary detection of early neoplastic lesions in BE, wide-field imaging techniques are required that allow detection of lesions in overview: to "red flag" areas of interest. As stated in current guidelines, advanced imaging techniques should be superimposed on high-resolution white light endoscopy (WLE) using high-definition (HD) systems [8, 21, 22].

Chromoendoscopy

In chromoendoscopy, stains are applied to the mucosa to improve the visualization of neoplastic lesions. Vital stains (e.g., methylene blue) are actively absorbed by the epithelium. Contrast stains (e.g., indigo carmine) accumulate in pits and grooves

along the epithelial surface, highlighting the superficial mucosal architecture (Fig. 5.3). Early studies on methylene blue chromoendoscopy suggested an increased detection of early neoplasia [23], yet a recent meta-analysis of 9 studies showed that there is no incremental yield for methylene blue chromoendoscopy over standard WLE [24]. Acetic acid is an inexpensive agent that increases the contrast of the mucosal pattern (Fig. 5.3). Recent publications have suggested that acetic acid may be beneficial for identification of early neoplasia [25, 26]. However, no randomized (cross-over) controlled studies have been performed comparing acetic acid to standard practice and other studies have questioned the additional value of acetic acid over HD-WLE [27].

Chromoendoscopy techniques are not widely used in Barrett's endoscopy: it is questionable if they really increase the detection of early neoplasia over HD-WLE, many endoscopists consider chromoendoscopy a cumbersome procedure, and correct application of dyes and interpretation of the images are operator dependent.

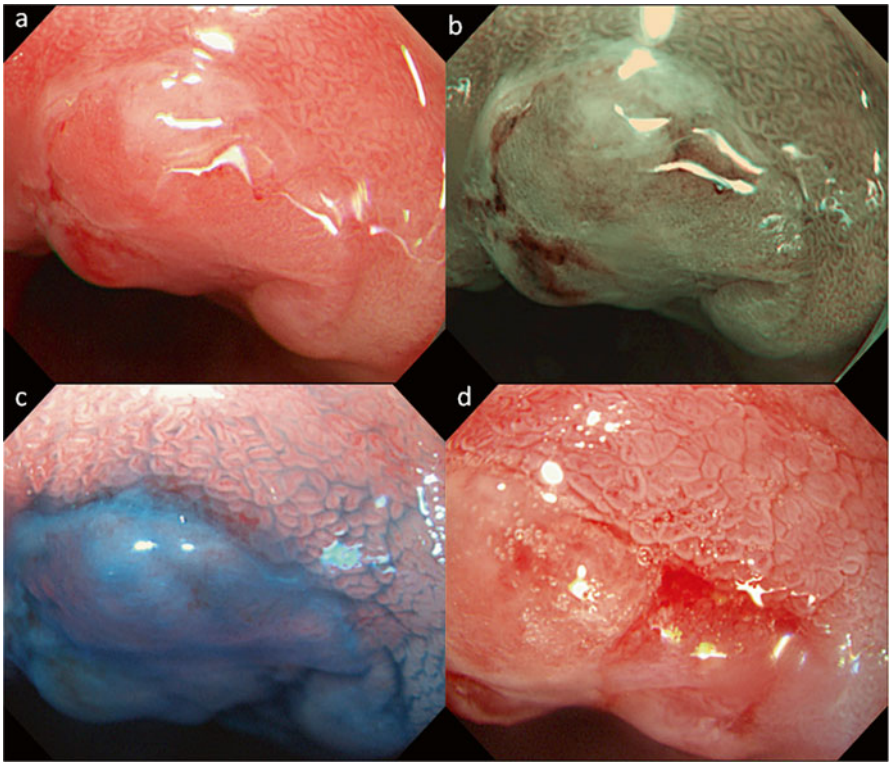

Fig. 5.3 Image of an early neoplastic lesion in the distal esophagus, (**a**) with high resolution white light endoscopy, (**b**) with narrow band imaging, (**c**) after indigo carmine spraying, (**d**) after acetic acid spraying

Optical and Digital Chromoendoscopy Techniques

These techniques improve the visualization of mucosal morphology without the use of dyes. This can be done with *preprocessing* techniques—optical chromoendoscopy—such as narrow band imaging (NBI; Olympus, Tokyo, Japan), or blue laser imaging (BLI; Fujifilm, Tokyo, Japan). The mucosal imaging is enhanced by using blue light, which only penetrates superficially into the tissue and causes less scattering. In addition, blue light encompasses the maximum absorption wavelength of hemoglobin, which results in better visualization of vascular structures [28].

Digital chromoendoscopy techniques that are based on *postprocessing* (Fujifilm intelligent chromo-endoscopy (FICE; Fujifilm, Tokyo, Japan) and i-scan (Pentax, Tokyo Japan)) use normal white light excitation. The reflected image is then reprocessed by a proprietary algorithm. In our opinion, preprocessing techniques have a better signal-to-noise ratio, resulting in images with a higher resolution and brightness compared to postprocessing techniques (Fig. 5.4).

Most studies on optical chromoendoscopy techniques in Barrett's esophagus have used NBI. Regular mucosal and vascular NBI patterns have been shown to

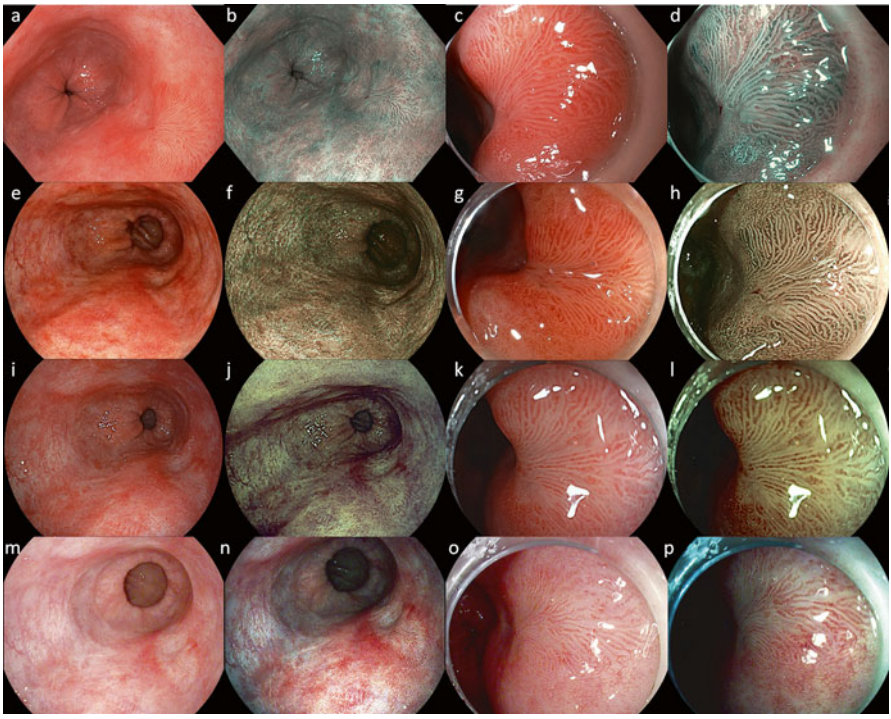

Fig. 5.4 Overview and detailed images of a neoplastic lesions in a Barrett's esophagus: Olympus high resolution white light endoscopy (**a, c**) and narrow band imaging (**b, d**); Fujinon white light endoscopy (**e, g**) and blue light imaging (**f, h**); Fujinon white light endoscopy (**i, k**) and fujinon intelligent chromoendoscopy (**j, l**); Pentax white light endoscopy (**m, o**) and iSCAN (**n, p**)

correlate with nondysplastic BE, while irregular features are associated with early neoplasia [28, 29].

The yield of NBI for the detection of early neoplasia has been investigated in three randomized studies. Kara et al. compared HD-WLE *plus* NBI to HD-WLE *plus* indigo carmine chromoendoscopy in a randomized cross-over design [11]. NBI and indigo carmine both increased the targeted detection of neoplastic lesions, but all patients with neoplasia were already diagnosed with HD-WLE. Wolfsen et al. suggested that NBI increases the detection of patients with early neoplasia over *standard* resolution WLE [19]. The tandem endoscopy design of this study, however, was biased because standard WLE endoscopy was performed by general endoscopists and compared to HD-WLE plus NBI inspection performed by endoscopists with experience in the detection of early Barrett's neoplasia [30]. Finally, a recent randomized cross-over study compared HD-WLE plus random biopsies to NBI with targeted biopsies only [31]. The authors conclude that although both modalities detected a comparable number of patients and lesions with early neoplasia, NBI may reduce the number of biopsies taken during Barrett's surveillance and thus add to its efficacy and (cost-) effectiveness. A drawback of this study was the relative low prevalence of early neoplasia.

Optical chromoendoscopy techniques offer a more detailed inspection of the mucosal morphology than HD-WLE, but whether this translates into clinically relevant information is yet unknown. After the initial enthusiasm, subsequent clinical studies have not provided new insights in detection of neoplasia by NBI [32, 33].

Autofluorescence Imaging

Autofluorescence imaging (AFI) is based on the principle that certain endogenous substances, such as nicotinamide adenine dinucleotide and collagen emit light of longer wavelengths when excited with light of short wavelength. Spectroscopy studies have shown that Barrett's neoplasia has a different autofluorescence spectrum compared to nonneoplastic Barrett's mucosa [34, 35]. These findings led to the development of wide-field autofluorescence imaging, that was integrated with HD-WLE and NBI into an "endoscopic trimodal imaging" (ETMI) system (Fig. 5.5).

In uncontrolled studies, AFI increased the detection of early neoplasia, while NBI reduced the false-positive rate associated with AFI [12]. However, two subsequent randomized crossover trials, comparing ETMI to standard resolution WLE, failed to show superiority of ETMI [36, 37]. In these studies, AFI again significantly increased the targeted detection of areas with neoplasia that were inconspicuous with WLE, but the strategy of only obtaining targeted biopsies after ETMI inspection was found to be inferior to standard WLE plus random biopsies.

The finding that AFI improves the targeted detection of neoplasia may be clinically relevant in two ways. First, there relevance from a *diagnostic* perspective: if the AFI detected lesion is the only neoplastic lesion identified during the endoscopy and all random biopsies are negative, AFI "upstages" the neoplastic status of the

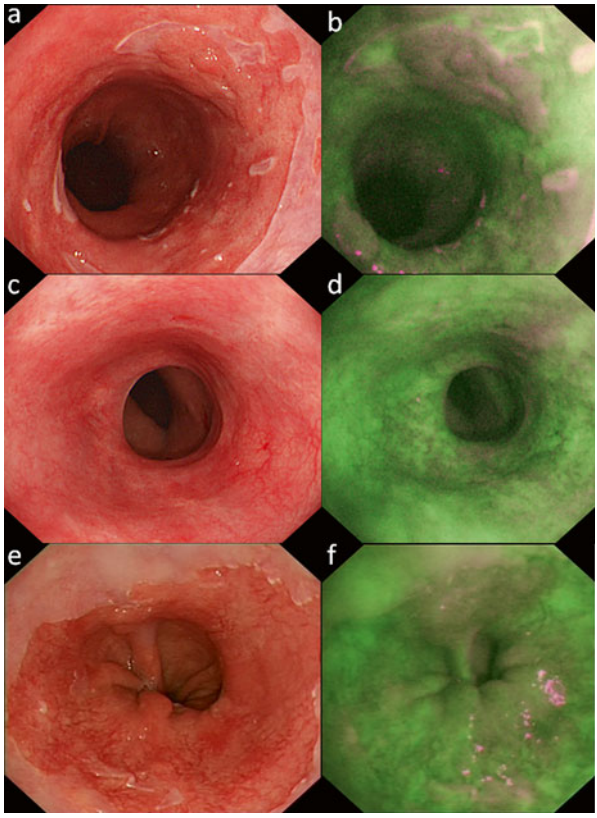

Fig. 5.5 Three examples of a neoplastic lesions in Barrett's esophagus, with white light endoscopy (WLE; **a, c, e**) and autofluorescence imaging (AFI; **b, d, f**). In (**c**, **d**), the lesion is hard to detect with WLE, but can be clearly appreciated with AFI. In (**e**, **f**) the lesion is located at the gastric folds, which makes the AFI interpretation difficult, resulting in high false-positive rates for AFI

patient. Second, there is relevance from a *therapeutic* perspective. In patients with an indication for endoscopic treatment, visible lesions should be resected and not ablated [8, 38]. AFI detected lesions may have therapeutic relevance if endoscopic resection of the lesion shows histology that changes the management from an endoscopic to a surgical approach. A recent study found that AFI detected lesions rarely lead to diagnostic upstaging of neoplasia or a change in the therapeutic approach. Neoplastic lesions that direct the choice of therapy are virtually always found with HD-WLE inspection only [39]. This is in line with previous observations in patients who were treated with stepwise endoscopic resection of the whole Barrett's segment: after endoscopic resection of the most suspicious lesion detected with HD-WLE, subsequent resections of the remaining Barrett's segment did not lead to histological upstaging of the neoplasia [40, 41].

Recently, third-generation AFI was introduced with a dual-band autofluorescence algorithm. The hypothesis was that this algorithm specifically targets fluorescent changes in neoplastic cells, yet initial feasibility studies have yielded disappointing results [42].

Real-Time Diagnosis and Decision Making

After the detection of suspicious lesions, advanced imaging techniques might be able to confirm the diagnosis of neoplasia without the need for histological evaluation, allowing real-time diagnosis and decision making during endoscopy.

Optical Chromoendoscopy

Optical chromoendoscopy enables detailed inspection of mucosal and vascular structures. However, multiple studies with different modalities have shown that so far these techniques do not allow a reliable distinction between neoplastic and non-neoplastic lesions [27, 36, 37, 43, 44].

Confocal Laser Endomicroscopy

Confocal laser endomicroscopy (CLE) has the potential of real-time histology during endoscopy. Probe-based CLE (pCLE) and integrated CLE (iCLE) have been studied in the colon, stomach, and esophagus [45]. Both techniques differ significantly in a practical sense: pCLE can be performed in combination with HR-WLE and other red-flag techniques, yet has a lower resolution and frame rate compared to iCLE. With iCLE, high resolution images can be obtained, while leaving room in the accessory channel for a biopsy forceps. However, the maneuverability of the stiff iCLE scope tip is limited and the system lacks HD-WLE. CLE has demonstrated good performance in predicting the presence of neoplasia in Barrett's esophagus [46, 47]. Moreover, HR-WLE in combination with pCLE was shown to increase the detection of early neoplasia, compared to HR-WLE alone [47]. With a sensitivity of 68 %, the performance of pCLE is limited. A promising benefit of CLE is the possible reduction of the number of random biopsies taken, by sampling only areas suspicious on CLE [48]. However, obtaining good quality CLE images is technically challenging, CLE equipment is expensive, and exogenous contrast agents are required. More importantly, the relevance of real-time diagnosis, risk stratification, and decision making during Barrett's endoscopies is questionable. In the presence of visible abnormalities on HD-WLE, few endoscopists will withhold taking biopsies based on CLE or another real-time diagnosis technique: the pretest likelihood of neoplasia is so high that neoplasia cannot be excluded based on a negative test result [37, 49]. Second, immediate decision making based on real-time diagnosis is neither practical nor ethical: patients need to be consented for endoscopic therapy and according to guidelines this should be centralized in high-volume centers, which generally implies referral to a different hospital [4, 22].

Optical Coherence Tomography

Optical coherence tomography (OCT) works analogous to ultrasound utilizing light waves instead of sound waves to form two-dimensional images based on differences in optical scattering of tissue structures. OCT is capable of generating cross-sectional images of tissues in real time with a resolution comparable to low-power microscopy. This direct optical diagnosis would guide the endoscopist in targeting suspicious areas and will avoid (numerous) random biopsies. Previous studies have suggested that OCT may differentiate between normal squamous mucosa, Barrett's epithelium, and HGD/EAC [50–55]. The clinical utility of first-generation OCT systems, however, was hampered by slow acquisition rates and small scanning areas. With the development of second-generation OCT, termed optical frequency domain imaging (OFDI), it is now possible to perform high resolution, high-speed acquisition of large luminal surfaces [56]. Recently, a balloon-based system incorporating OFDI was introduced: volumetric laser endomicroscopy (VLE) (NVisionVLE™, NinePoint Medical, Cambridge, MA, USA). This system provides a 6 cm long circumferential scan of the esophagus with a depth of 3 mm, in 96 s. Preliminary studies showed that OFDI provides a clear visualization of anatomic layers and vascular network of the esophagus and suggested that specific OFDI characteristics correlate with neoplasia [57–59]. In addition, relevant lesions visible on OFDI can be marked directly with laser [60]. OFDI, and specifically the VLE system, therefore has the potential for detection and delineation of early neoplastic lesions in BE.

Recommendations for Current Clinical Practice

Endoscopic Surveillance

Most surveillance endoscopies are performed in community hospitals. In this setting, the prevalence of early neoplasia is low (i.e., <5 %) and therefore endoscopists are generally not familiar with the endoscopic appearance of early Barrett's neoplasia [61, 62].

In surveillance settings, detection of early neoplasia can be significantly improved by the use of HD-WLE and implementation of the three rules for detection of early neoplastic lesions as abovementioned.

In our opinion, advanced imaging techniques have little clinical relevance for Barrett's surveillance, since the use of HD-WLE and adherence to the three rules for detection will detect the majority of neoplastic cases. The potential value of advanced imaging techniques is therefore small and with such a low pretest likelihood most techniques will suffer from unacceptably high false-positive rates [11, 36, 37]. Finally, advanced imaging techniques are costly, not widely available in community hospitals and require endoscopic expertise that may not be uniformly available.

Workup and Treatment of Early Neoplasia

Workup and treatment of early Barrett's neoplasia should be centralized in tertiary referral centers [4, 22]. Here, the prevalence of early neoplasia is much higher (i.e., >25 %) and procedures are performed under optimal circumstances by expert endoscopists. The workup endoscopy serves the following purposes: (1) confirmation of the referral diagnosis and indication for treatment; (2) visible lesions requiring endoscopic resection (instead of ablation) have to be detected and staged.

To confirm the referral diagnosis and indication for treatment, advanced imaging techniques have limited value: HD-WLE and the "detection essentials" generally suffice. Compared to surveillance settings, workup endoscopies are performed under better circumstances. The procedures are generally performed on a dedicated endoscopy program by an endoscopy team with experience in detection and treatment of Barrett's neoplasia. More importantly, the team is aware of the neoplastic status of the Barrett's segment, based on the referral information.

Advanced imaging techniques have limited value for detection of lesions that require endoscopic resection. These lesions will virtually always be detected on HD-WLE by the expert team performing the workup endoscopy. Advanced imaging techniques may indeed detect additional flat lesions, inconspicuous with WLE, but these are clinically of limited significance since they harbor only flat mucosal neoplasia that will be effectively eradicated by ablation [39].

Staging of early neoplastic lesions implies evaluation of invasion depth. Advanced imaging techniques have limited value in distinguishing mucosal from submucosal cancers. Several studies have demonstrated that EUS provides no clinically relevant information over endoscopic inspection with HD-WLE [63–65]. CLE has a limited scanning depth and is therefore not suited for assessment of depth invasion. Histological evaluation of the resected specimen not only provides the ultimate proof of invasion depth, but also allows diagnosis of poorly differentiated cancers and lymphatic invasion, features that are virtually impossible to detect otherwise.

Before endoscopic resection, lesions have to be delineated from the surrounding mucosa. Advanced imaging techniques may facilitate delineation of lesions prior to endoscopic resection, but formal studies are lacking for most techniques [46, 60, 66, 67]. Delineation of lesions should meet the purpose of endoscopic resection of Barrett's neoplasia: removal of the most involved area to finalize staging and rendering the Barrett's segment flat for subsequent ablation therapy [8, 68]. In our opinion, NBI is superior to HD-WLE for this purpose. Detailed inspection with NBI allows for identification of the demarcation line (Fig. 5.6), separating the area with an irregular mucosal and vascular pattern from its normal surroundings, like the delineation performed for resection of early gastric neoplasia [69]. Optical chromoendoscopy techniques also have an important role in the follow-up of patients after ablation, allowing for the detection of small residual islands of Barrett's mucosa that are easily overlooked with HD-WLE. Recent studies suggest that detailed inspection with NBI of the post-RFA neo-squamous epithelium is probably more useful than obtaining random biopsies [70].

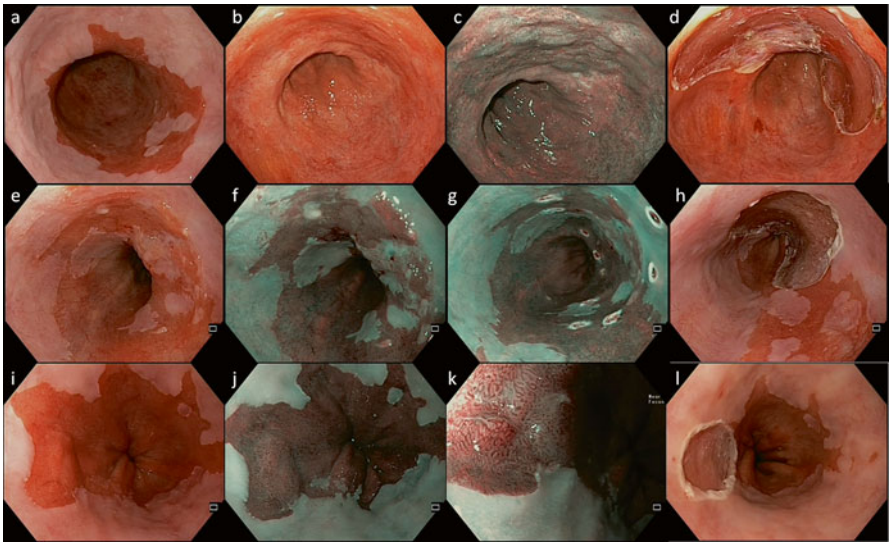

Fig. 5.6 Narrow band imaging (NBI) not only facilitates the evaluation of the mucosal and vascular patterns, but also enhances visualization of the mucosal relief, a distinct and recognizable feature of NBI (**c, f, j, k**). This can be observed when comparing to the white light images (**a, b, e, i**). While the mucosal and vascular patterns may be regular, the relief can be used to assess the extension of the lesion to direct the delineation (**g**) prior to endoscopic resection (**d, h, l**)

Future Perspectives

Detection of early neoplasia can be improved by optimizing the endoscopists' recognition of "the face of Barrett's neoplasia" but there are few tools to aid this [8, 21]. The international workgroup for the classification of esophagitis [13, 71] is working on a training program for "Barrett's esophagus related neoplasia" (BORN-project) that will be dispersed to the gastroenterology community.

In the near future, molecular markers may enable us to predict which patients will develop neoplasia well before morphological changes can be observed histologically. Advanced imaging techniques may aid this risk stratification by detecting areas containing relevant biomarkers [72, 73].

Another interesting development is the optical detection of (sub)structural abnormalities that may be correlated with an underlying field carcinogenesis [74]. Spectroscopy and OCT may provide quantitative measurements of tissue allowing direct optical diagnosis of early neoplasia or risk stratification based on the presence of field carcinogenesis [75].

Practice Points

- The use of advanced imaging techniques does not significantly increase the diagnostic yield of early neoplasia compared with high-definition white light endoscopy (HD-WLE) with random biopsy analysis.

- For the detection of subtle abnormalities in the esophagus, a careful and thorough inspection following a systematic approach is imperative.
- For primary detection of early neoplastic lesions in BE, advanced imaging techniques should be superimposed on WLE.
- Lesions that require resection are almost always detected by HD-WLE, although advanced imaging techniques can detect additional flat lesions.
- Workup and treatment of early Barrett's neoplasia should be centralized in tertiary referral centers.
- Advanced imaging techniques may facilitate delineation of lesions prior to endoscopic resection, but formal studies are lacking for most techniques.

Research Agenda

- Advanced imaging techniques may aid the risk stratification of future neoplasia development by detecting areas containing relevant biomarkers.
- NBI features relevant for detection in overview need to be established.
- Optical techniques like OCT and spectroscopy providing quantitative measurements of tissue need further investigation to demonstrate the ability of direct optical diagnosis of early neoplasia or risk stratification based on the presence of field carcinogenesis.

References

1. Thrift AP, Whiteman DC. The incidence of esophageal adenocarcinoma continues to rise: analysis of period and birth cohort effects on recent trends. Ann Oncol Off J Eur Soc Med Oncol. 2012;23(12):3155–62.
2. De Boer AGEM, van Lanschot JJB, van Sandick JW, Hulscher JBF, Stalmeier PFM, de Haes JCJM, et al. Quality of life after transhiatal compared with extended transthoracic resection for adenocarcinoma of the esophagus. J Clin Oncol Off J Am Soc Clin Oncol. 2004;22(20):4202–8.
3. Hornick JL, Odze RD. Neoplastic precursor lesions in Barrett's esophagus. Gastroenterol Clin North Am. 2007;36(4):775–96. v.
4. Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. Am J Gastroenterol. 2008;103(3):788–97.
5. Tschanz ER. Do 40 % of patients resected for barrett esophagus with high-grade dysplasia have unsuspected adenocarcinoma? Arch Pathol Lab Med. 2005;129(2):177–80.
6. Peters FP, Curvers WL, Rosmolen WD, de Vries CE, Ten Kate FJW, Krishnadath KK, et al. Surveillance history of endoscopically treated patients with early Barrett's neoplasia: nonadherence to the Seattle biopsy protocol leads to sampling error. Dis Esophagus Off J Int Soc Dis Esophagus. 2008;21(6):475–9.
7. Boerwinkel DF, Swager A, Curvers WL, Bergman JJ. The clinical consequences of advanced imaging techniques in Barrett's esophagus. Gastroenterology. Elsevier, Inc; 2014:1–8.
8. Curvers WL, Bansal A, Sharma P, Bergman JJ. Endoscopic work-up of early Barrett's neoplasia. Endoscopy. 2008;40(12):1000–7.
9. Bruno MJ. Magnification endoscopy, high resolution endoscopy, and chromoscopy; towards a better optical diagnosis. Gut. 2003;52(Suppl 4(Ccd)):iv7–11.
10. Subramanian V, Ragunath K. Advanced endoscopic imaging: a review of commercially available technologies. Clin Gastroenterol Hepatol. Elsevier, Inc; 2014;1–10.

11. Kara MA, Peters FP, Rosmolen WD, Krishnadath KK, Ten Kate FJW, Fockens P, et al. High-resolution endoscopy plus chromoendoscopy or narrow-band imaging in Barrett's esophagus: a prospective randomized crossover study. Endoscopy. 2005;37(10):929–36.
12. Curvers WL, Singh R, Song L-MW-K, Wolfsen HC, Ragunath K, Wang K, et al. Endoscopic tri-modal imaging for detection of early neoplasia in Barrett's oesophagus: a multi-centre feasibility study using high-resolution endoscopy, autofluorescence imaging and narrow band imaging incorporated in one endoscopy system. Gut. 2008;57(2):167–72.
13. Sharma P, Dent J, Armstrong D, Bergman JJGHM, Gossner L, Hoshihara Y, et al. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. Gastroenterology. 2006;131(5):1392–9.
14. Alvarez Herrero L, Curvers WL, van Vilsteren FGI, Wolfsen H, Ragunath K, Wong Kee Song L-M, et al. Validation of the Prague C&M classification of Barrett's esophagus in clinical practice. Endoscopy. © Georg Thieme Verlag KG; 2013;45(11):876–82.
15. Pech O, Gossner L, Manner H, May A, Rabenstein T, Behrens A, et al. Prospective evaluation of the macroscopic types and location of early Barrett' s neoplasia in 380 lesions. 2007;588–93.
16. Anonymous. The Paris endoscopic classification of superficial neoplastic lesions: esophagus, stomach, and colon: November 30 to December 1, 2002. Gastrointest Endosc. Mosby; 2003;58(6 Suppl):S3–43.
17. Endoscopic Classification Review Group. Update on the paris classification of superficial neoplastic lesions in the digestive tract. Endoscopy Thieme. 2005;37(6):570–8.
18. Peters FP, Brakenhoff KPM, Curvers WL, Rosmolen WD, Fockens P, Ten Kate FJW, et al. Histologic evaluation of resection specimens obtained at 293 endoscopic resections in Barrett's esophagus. Gastrointest Endosc. 2008;67(4):604–9.
19. Wolfsen HC, Crook JE, Krishna M, Achem SR, Devault KR, Bouras EP, et al. Prospective, controlled tandem endoscopy study of narrow band imaging for dysplasia detection in Barrett's esophagus. Gastroenterology. 2008;135(1):24–31. Elsevier.
20. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association medical position statement on the management of Barrett's esophagus. Gastroenterology. Elsevier Inc.; 2011;140(3):1084–91.
21. Bennett C, Vakil N, Bergman J, Harrison R, Odze R, Vieth M, et al. Consensus statements for management of Barrett's dysplasia and early-stage esophageal adenocarcinoma, based on a Delphi process. Gastroenterology. Elsevier Inc.; 2012;143(2):336–46.
22. Sharma P, Savides TJ, Canto MI, Corley DA, Falk GW, Goldblum JR, et al. The American Society for gastrointestinal endoscopy PIVI (preservation and incorporation of valuable endoscopic innovations) on imaging in Barrett's esophagus. Gastrointest Endosc. 2012;76(2):252–4.
23. Canto MI, Setrakian S, Willis J, Chak A, Petras R, Powe NR, et al. Methylene blue-directed biopsies improve detection of intestinal metaplasia and dysplasia in Barrett's esophagus. Gastrointest Endosc. 2000;51(5):560–8.
24. Ngamruengphong S, Sharma VK, Das A, American Society for Gastrointestinal Endoscopy. Diagnostic yield of methylene blue chromoendoscopy for detecting specialized intestinal metaplasia and dysplasia in Barrett's esophagus: a meta-analysis. Gastrointest Endosc. 2009;69(6):1021–8.
25. Longcroft-Wheaton G, Duku M, Mead R, Poller D, Bhandari P. Acetic acid spray is an effective tool for the endoscopic detection of neoplasia in patients with Barrett's esophagus. Clin Gastroenterol Hepatol. Elsevier Inc.; 2010;8(10):843–7.
26. Bhandari P, Kandaswamy P, Cowlishaw D, Longcroft-Wheaton G. Acetic acid-enhanced chromoendoscopy is more cost-effective than protocol-guided biopsies in a high-risk Barrett's population. Dis Esophagus. 2012;25(5):386–92.
27. Curvers W, Baak L, Kiesslich R, Van Oijen A, Rabenstein T, Ragunath K, et al. Chromoendoscopy and narrow-band imaging compared with high-resolution magnification endoscopy in Barrett's esophagus. Gastroenterology. 2008;134(3):670–9.
28. Gono K, Obi T, Yamaguchi M, Ohyama N, Machida H, Sano Y, et al. Appearance of enhanced tissue features in narrow-band endoscopic imaging. J Biomed Opt. 2004;9(3):568–77.

29. Kara MA, Ennahachi M, Fockens P, ten Kate FJW, Bergman JJGHM. Detection and classification of the mucosal and vascular patterns (mucosal morphology) in Barrett's esophagus by using narrow band imaging. Gastrointest Endosc. 2006;64(2):155–66.
30. Curvers WL, Bergman JJGHM. Multimodality imaging in Barrett's esophagus: looking longer, seeing better, and recognizing more. Gastroenterology. 2008;135(1):297–9.
31. Sharma P, Hawes RH, Bansal A, Gupta N, Curvers W, Rastogi A, et al. Standard endoscopy with random biopsies versus narrow band imaging targeted biopsies in Barrett's oesophagus: a prospective, international, randomised controlled trial. Gut. 2013;62:15–21.
32. Jayasekera C, Taylor ACF, Desmond PV, Macrae F, Williams R, Royal T, et al. Added value of narrow band imaging and confocal laser endomicroscopy in detecting Barrett' s esophagus neoplasia. Endoscopy. 2012;44:1089–95.
33. Singh R, Shahzad MA, Tam W, Goda K, Yu LHK, Fujishiro M, et al. Preliminary feasibility study using a novel narrow-band imaging system with dual focus magnification capability in Barrett's esophagus: is the time ripe to abandon random biopsies? Dig Endosc Off J Japan Gastroenterol Endosc Soc. 2013;25 Suppl 2:151–6.
34. Georgakoudi I, Jacobson BC, Van Dam J, Backman V, Wallace MB, Müller MG, et al. Fluorescence, reflectance, and light-scattering spectroscopy for evaluating dysplasia in patients with Barrett's esophagus. Gastroenterology. 2001;120(7):1620–9.
35. Panjehpour M, Overholt BF, Vo-Dinh T, Haggitt RC, Edwards DH, Buckley 3rd FP. Endoscopic fluorescence detection of high-grade dysplasia in Barrett's esophagus. Gastroenterology. 1996;111(1):93–101.
36. Curvers WL, Herrero LA, Wallace MB, Wong Kee Song L-M, Ragunath K, Wolfsen HC, et al. Endoscopic tri-modal imaging is more effective than standard endoscopy in identifying early-stage neoplasia in Barrett's esophagus. Gastroenterology. Elsevier Inc.; 2010;139(4):1106–14.
37. Curvers WL, van Vilsteren FG, Baak LC, Böhmer C, Mallant-Hent RC, Naber AH, et al. Endoscopic trimodal imaging versus standard video endoscopy for detection of early Barrett's neoplasia: a multicenter, randomized, crossover study in general practice. Gastrointest Endosc. 2011;73(2):195–203.
38. Evans JA, Early DS, Fukami N, Ben-Menachem T, Chandrasekhara V, Chathadi KV, et al. The role of endoscopy in Barrett's esophagus and other premalignant conditions of the esophagus. Gastrointest Endosc. 2012;76(6):1087–94.
39. Boerwinkel D, Holz JA, Kara MA, Meijer SL, Wallace MB, Wong Kee Song L-M, et al. Effects of autofluorescence imaging on detection and treatment of early neoplasia in patients with Barrett's esophagus. Clin Gastroenterol Hepatol. 2014;12(5):774–81.
40. Peters FP, Kara MA, Rosmolen WD, ten Kate FJW, Krishnadath KK, van Lanschot JJB, et al. Stepwise radical endoscopic resection is effective for complete removal of Barrett's esophagus with early neoplasia: a prospective study. Am J Gastroenterol. 2006;101(7):1449–57.
41. Pouw RE, Seewald S, Gondrie JJ, Deprez PH, Piessevaux H, Pohl H, et al. Stepwise radical endoscopic resection for eradication of Barrett's oesophagus with early neoplasia in a cohort of 169 patients. Gut. 2010;59(9):1169–77.
42. Boerwinkel DF, Holz JA, Aalders MCG, Visser M, Meijer SL, Van Berge Henegouwen MI, et al. Third-generation autofluorescence endoscopy for the detection of early neoplasia in Barrett's esophagus: a pilot study. Dis Esophagus. 2014;27(3):276–84.
43. Curvers WL, Bohmer CJ, Mallant-Hent RC, Naber AH, Ponsioen CI, Ragunath K, et al. Mucosal morphology in Barrett's esophagus: interobserver agreement and role of narrow band imaging. Endoscopy. 2008;40(10):799–805.
44. Singh M, Bansal A, Curvers WL, Kara MA, Wani SB, Alvarez Herrero L, et al. Observer agreement in the assessment of narrowband imaging system surface patterns in Barrett's esophagus: a multicenter study. Endoscopy. 2011;43(9):745–51.
45. Li W-B, Zuo X-L, Li C-Q, Zuo F, Gu X-M, Yu T, et al. Diagnostic value of confocal laser endomicroscopy for gastric superficial cancerous lesions. Gut. 2011;60(3):299–306.
46. Kiesslich R, Gossner L, Goetz M, Dahlmann A, Vieth M, Stolte M, et al. In vivo histology of Barrett's esophagus and associated neoplasia by confocal laser endomicroscopy. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2006;4(8):979–87.

47. Sharma P, Meining AR, Coron E, Lightdale CJ, Wolfsen HC, Bansal A, et al. Real-time increased detection of neoplastic tissue in Barrett's esophagus with probe-based confocal laser endomicroscopy: final results of an international multicenter, prospective, randomized, controlled trial. Gastrointest Endosc. 2011;74(3):465–72.
48. Dunbar KB, Okolo PI, Montgomery E, Canto MI. Confocal laser endomicroscopy in Barrett's esophagus and endoscopically inapparent Barrett's neoplasia: a prospective, randomized, double-blind, controlled, crossover trial.pdf. Gastrointest Endosc. 2009;70(4):645–54.
49. Boerwinkel DF, Shariff MK, di Pietro M, Holz JA, Aalders MC, Curvers WL, et al. Fluorescence imaging for the detection of early neoplasia in Barrett's esophagus: old looks or new vision? Eur J Gastroenterol Hepatol. 2014;26(7):691–8.
50. Evans JA, Poneros JM, Bouma BE, Bressner J, Elkan F, Shishkov M, et al. Optical coherence tomography to identify intramucosal carcinoma and high-grade dysplasia in Barrett's esophagus. Clin Gastroenterol Hepatol. 2006;4(1):38–43.
51. Evans JA, Bouma BE, Bressner J, Shishkov M. Identifying intestinal metaplasia at the squamocolumnar junction by using optical coherence tomography. Gastrointest Endosc NIH Public Access. 2007;65(1):50–6.
52. Cobb MJ, Hwang JH, Upton MP, Chen Y, Oelschlager BK, Wood DE, American Society for Gastrointestinal Endoscopy, et al. Imaging of subsquamous Barrett's epithelium with ultrahigh-resolution optical coherence tomography: a histologic correlation study. Gastrointest Endosc. 2010;71(2):223–30.
53. Isenberg G, Sivak MV, Chak A, Wong RCK, Willis JE, Wolf B, et al. Accuracy of endoscopic optical coherence tomography in the detection of dysplasia in Barrett's esophagus: a prospective, double-blinded study. Gastrointest Endosc. 2005;62(6):825–31.
54. Sivak M V, Kobayashi K, Joseph A, Rollins AM, Wong RCK, Isenberg GA, et al. High-resolution endoscopic imaging of the GI tract using optical coherence tomography. Gastrointest Endosc. 2000;51(4).
55. Poneros JM, Brand S, Bouma BE, Tearney GJ, Compton CC, Nishioka NS, American Gastroenterological Association. Diagnosis of specialized intestinal metaplasia by optical coherence tomography. Gastroenterology. 2001;120(1):7–12.
56. Bouma BE, Yun SH, Vakoc BJ, Suter MJ, Tearney GJ. Fourier-domain optical coherence tomography: recent advances toward clinical utility. Curr Opin Biotechnol. 2009;20(1):111–8.
57. Vakoc BJ, Shishko M, Yun SH, Oh W, Tearney GJ, Bouma BE. Comprehensive esophageal microscopy by using optical frequency-domain imaging (with video). Gastrointest Endosc. 2007;65(6):898–905.
58. Yun SH, Tearney GJ, Vakoc BJ, Shishkov M, Oh WY, Desjardins AE, et al. Comprehensive volumetric optical microscopy in vivo. Nat Med. 2006;12:1429–33.
59. Suter MJ, Vakoc BJ, Yachimski P, Shishkov M, Lauwers GY, Mino-Kenudson M, et al. Comprehensive microscopy of the esophagus in human patients with optical frequency domain imaging. Array, editor. Gastrointest Endosc. 2008;68(4):745–53. Springer.
60. Suter MJ, Jillella PA, Vakoc BJ, Halpern EF, Mino-kenudson M, Lauwers GY, et al. Image-guided biopsy in the esophagus through comprehensive optical frequency domain imaging and laser marking: a study in living swine. Gastrointest Endosc. 2010;71(2):346–53.
61. Shaheen NJ, Crosby MA, Bozymski EM, Sandler RS. Is there publication bias in the reporting of cancer risk in Barrett's esophagus? Gastroenterology. 2000;119(2):333–8.
62. Hvid-Jensen F, Pedersen L, Asbjorn MD, Sørensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011;365(15):1375–83.
63. Pouw RE, Heldoorn N, Herrero LA, ten Kate FJW, Visser M, Busch OR, et al. Do we still need EUS in the workup of patients with early esophageal neoplasia? A retrospective analysis of 131 cases. Gastrointest Endosc. 2011;73(4):662–8.
64. Pech O, Günter E, Dusemund F, Origer J, Lorenz D, Ell C. Accuracy of endoscopic ultrasound in preoperative staging of esophageal cancer: results from a referral center for early esophageal cancer. Endoscopy. 2010;42(6):456–61.

65. May A, Günter E, Roth F, Gossner L, Stolte M, Vieth M, et al. Accuracy of staging in early oesophageal cancer using high resolution endoscopy and high resolution endosonography: a comparative, prospective, and blinded trial. Gut. Copyright 2004 by Gut; 2004;53(5):634–40.
66. Thomas T, Singh R, Ragunath K. Trimodal imaging-assisted endoscopic mucosal resection of early Barrett's neoplasia. Surg Endosc. 2009;23(7):1609–13.
67. Kara MA, DaCosta RS, Streutker CJ, Marcon NE, Bergman JJGHM, Wilson BC. Characterization of tissue autofluorescence in Barrett's esophagus by confocal fluorescence microscopy. Dis Esophagus Off J Int Soc Dis Esophagus. 2007;20(2):141–50.
68. Pouw RE, Bergman JJGHM. Radiofrequency ablation for Barrett's esophagus, for whom and by whom? Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2013;11(10):1256–8.
69. Nagahama T, Yao K, Maki S, Yasaka M, Takaki Y, Matsui T, et al. Usefulness of magnifying endoscopy with narrow-band imaging for determining the horizontal extent of early gastric cancer when there is an unclear margin by chromoendoscopy (with video). Gastrointest Endosc. 2011;74(6):1259–67.
70. Phoa KN, Pouw RE, van Vilsteren FGI, Sondermeijer CMT, Ten Kate FJW, Visser M, et al. Remission of Barrett's esophagus with early neoplasia 5 years after radiofrequency ablation with endoscopic resection: a Netherlands cohort study. Gastroenterology. 2013;145(1): 96–104.
71. Lundell LR, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. Gut. 1999;45(2):172–80.
72. Boerwinkel DF, Di Pietro M, Liu X, Shariff MK, Lao-Sirieix P, Walker CE, et al. Endoscopic TriModal imaging and biomarkers for neoplasia conjoined: a feasibility study in Barrett's esophagus. Dis Esophagus. 2014;27(5):435.
73. Shariff KM, Pietro M, Boerwinkel DF, Liu X, Lao-sirieix P, Walker EC, et al. Time: a prospective study combining endoscopic trimodal imaging and molecular endpoints to improve risk stratification in Barrett's esophagus (abstract). Gastroenterology. 2012;142(5):S-165.
74. Konda VJ, Cherkezyan L, Subramanian H, Wroblewski K, Damania D, Becker V, et al. Nanoscale markers of esophageal field carcinogenesis: potential implications for esophageal cancer screening. Endoscopy. 2013;45(12):983–8.
75. Swager A, de Bruin D, Faber D, Weusten B, Meijer S, Bergman J, et al. Quantitative analysis of volumetric laser endomicroscopy images with histological correlation of ex-vivo endoscopic resection specimens of Barrett's esophagus with and without early neoplasia. Gastroenterology. 2014;146(5):S-10.

Chapter 6
Endoscopic Treatment of Early Barrett's Neoplasia: Expanding Indications, New Challenges

Oliver Pech

Introduction

The incidence of Barrett's adenocarcinoma has shown a dramatic increase over the last decades and is still a fatal disease when diagnosed at an advanced stage. Increasing knowledge and awareness and also the technological development toward endoscopes with high resolution and excellent image quality lead to more diagnoses of the disease at an early stage when local treatment can be curative (Fig. 6.1).

Over the last 20 years endoscopic therapy of early Barrett's neoplasia has developed from an experimental treatment method that was rejected by many opinion leaders to a well-established first-line treatment method which is now recommended over surgery in most international guidelines [1–3]. Local endoscopic treatment in these patients is possible because the risk of lymph node metastases in low-grade, high-grade intraepithelial neoplasia (HGIN) or mucosal Barrett's cancer is almost nil.

Endoscopic Treatment Methods

Endoscopic (Mucosal) Resection Techniques

Endoscopic (mucosal) resection (ER or EMR) is usually performed with one of the "suck-and-cut" methods: Cap-ER (ER-C) or ER with a ligation device (ER-L)

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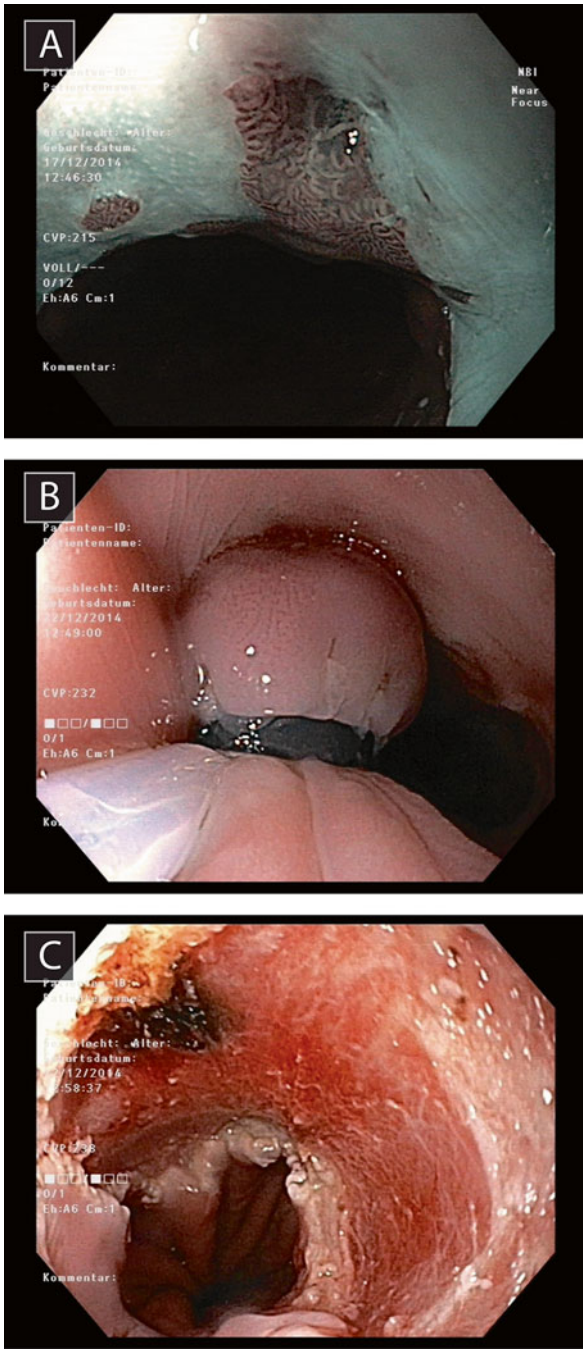

Fig. 6.1 (**a–c**) ER of a mucosal Barrett's adenocarcinoma with the ligation device

Cap-ER

The ER-C method was introduced by Dr. Inoue for resection of early esophageal squamous cell cancer [4]. First of all, the margins of the target lesion have to be marked with a distance of 5 mm with coagulation marks by using an APC probe or the tip of the snare. Prior ER-C submucosal injection of the lesion either with a saline–epinephrine solution or hyperosmotic solution underneath the target lesion prior resection should be performed in order to prevent perforation of the muscularis propria. Using the ER-C method a specially developed transparent plastic cap that is attached to the distal tip of the endoscope and dedicated crescent snare is preloaded into a rim at the distal end of the cap. It can be useful to tape the cap to the distal end of the endoscope in order to prevent slipping of the cap.

After injection, the lesion is sucked into the cap with the preloaded snare after which the snare is closed tightly. The tissue which has been sucked into the cap is then resected with a blended cutting current. The cap technique can be used for *en bloc* resection of lesions up to 15 mm but is also ideal for piece meal resection of larger lesions. For consecutive resections, the margin of the cap should be placed exactly at the margin of the previous resection site with only little overlap. This is done in order to prevent perforation (due to sucking the deeper muscle layer into the cap) but also to avoid leaving behind residual neoplastic tissue.

ER with a Ligation Device (ER-L)

Another widely used "suck-and-cut" technique is ER-L using either a device originally used for ligation of esophageal varices or a specially developed multiband mucosectomy (MBM) resection device where up to 6 rubber bands are preloaded and a snare can be advanced over the working channel without the need to demount the ligation device. This allows the endoscopist to perform piece meal resection of large areas without retracting the endoscope [5].

With the ER-L technique, the target lesion is sucked into the cylinder of the ligation device and a rubber band is then released to create a pseudopolyp with the rubber band tightly around its base. In contrast to ER-C, prior submucosal injection is usually not performed. Afterward the pseudopolyp containing the neoplastic tissue is resected with a snare. It is important that the snare is placed underneath the rubber band to achieve large resection specimens.

In a recently published study, the Wiesbaden group reported on the long-term results of ER in 1000 patients with mucosal Barrett's adenocarcinoma. After a follow-up of 57 months, 94 % of patients were in complete remission of HGIN or cancer. These excellent results clearly demonstrate the safety and efficacy of endoscopic treatment of early Barrett's neoplasia [6].

Endoscopic Submucosal Dissection

Endoscopic submucosal dissection (ESD) is a resection technique allowing the endoscopist to resect large neoplastic lesions *en bloc*. This facilitates histopathological analysis of the lateral margins of the specimen. ESD was introduced by Japanese endoscopists several years ago and has been adopted in some expert centers in the Western world. So far, there is only limited data on ESD in patients with HGIN and Barrett's adenocarcinoma. However, the results regarding the R0 resection rate—the ultimate goal of ESD—have been disappointing. Three European expert centers have published their experience and were able to perform a R0 resection defined as no HGIN or cancer at the lateral of basal margin in only 38.4–82 % [7–9]. The operation time of ESD is significantly longer and the complication rate is significantly higher than with conventional ER. When we take the excellent results of conventional ER into consideration, it is clear that currently there is no indication for the use of ESD in early Barrett's neoplasia outside of clinical studies.

Photodynamic Therapy

Photodynamic therapy (PDT) has been successfully used to treat early neoplasia in Barrett's esophagus for many more years. In the US, PDT had been regarded as the endoscopic treatment of choice until radiofrequency ablation became available. PDT has proven its efficacy in many prospective trials [10–17]. However, PDT has several disadvantages which have led to a dramatic decrease in its use: PDT with porfimer sodium is expensive and has a high complication rate. Photosensitivity and stricture formation in up to 30 % of patients are important drawbacks of this method [14, 16, 17]. Recent studies were able to identify the following risk factors for stricture formation: multiple PDT courses, longer length of Barrett's esophagus, presence of intramucosal carcinoma, ER before PDT, and prior history of esophageal stricture [16, 17]. The use of 5-aminolevulinic acid as a photosensitizer for PDT was associated with considerably fewer complications and showed promising short- and long-term results [10, 12]. In summary, PDT is no longer considered management option of choice for treatment of early Barrett's neoplasia.

Radiofrequency Ablation

Radiofrequency ablation (RFA) for treatment of neoplastic Barrett's epithelium has replaced PDT in all centers. For RFA a balloon catheter 3 cm in length with circular electrodes delivering the energy designed for circumferential ablation (HALO-360) is used for long segments of Barrett's epithelium. There are several focal devices available that fit over the tip of the endoscope or that can be advanced through the working channel and can be used for ablation of smaller areas. In a prospective randomized sham-controlled multicenter study from the US, it was shown that RFA of LGIN and HGIN has a significantly higher rate of complete remission of neoplasia

and intestinal metaplasia compared to proton pump inhibitor PPI treatment and follow-up alone. Those results were not surprising, but a HGIN eradication rate of 81 % after 1 year is too low to recommend this method alone for treatments of HGIN [18] (Fig. 6.2).

Like with all ablative methods, histological verification of the treated lesion (infiltration depth, differentiation grade, lymphovascular invasion, etc.) is not available. Thus, when treating incipient neoplasia, one potential problem is "undercalling" a neoplastic lesion after which the endoscopist may unknowingly ablate a submucosal carcinoma, or superficial lesion showing lymphatic spread. Therefore, all visible and detectable lesions within the Barrett's segment should be treated by endoscopic resection (and be sent for histopathological verification) and the residual Barrett's epithelium without visible lesions should be treated by RFA. The threshold for ER should be low for these patients in order to achieve histological confirmation.

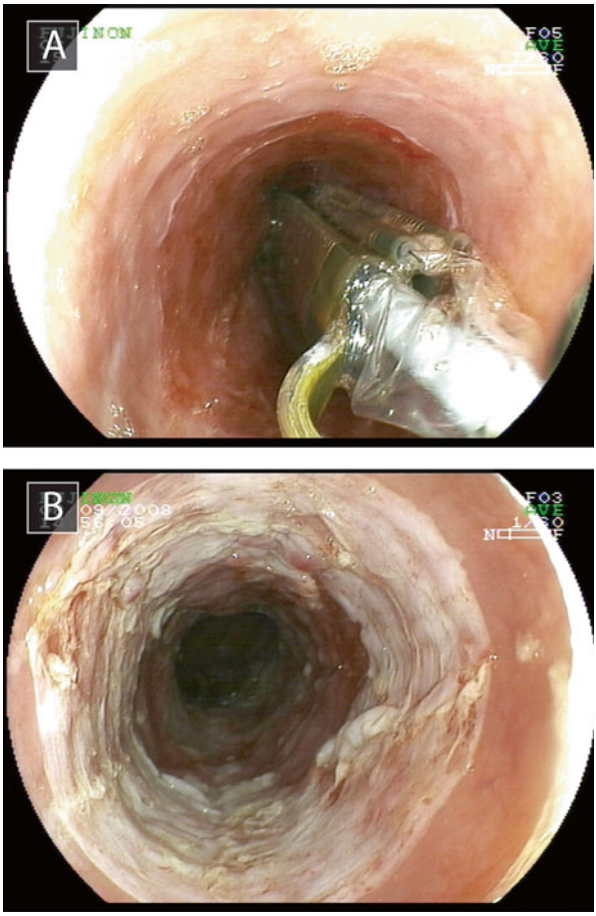

Fig. 6.2 (**a**, **b**) Radiofrequency ablation of a long-segment-Barrett's esophagus with the HALO 360° catheter after focal ER of an early Barrett's cancer

Compared to earlier data, recent studies report a disappointing complete remission rate and high recurrence rate of Barrett's mucosa after RFA [19, 20]. In a large series from three experienced US centers, complete remission of intestinal metaplasia was achieved in 56 % of patients only and recurrences of Barrett's mucosa were found in 33 % after complete ablation. Data from an UK registry report similar suboptimal results with complete remission of Barrett's mucosa and dysplasia in 62 and 81 % after 1 year, respectively. According to those data, it seems that results in daily practice are significantly worse than in rigorous prospective studies. Therefore, life-long follow-up is required even after complete removal of all neoplastic lesions and Barrett's mucosa.

The above described "two-step-concept" consisting of ER (step one) followed by ablation (step two) was investigated by a recent European prospective multicenter study where 132 patients with HGIN and early Barrett's adenocarcinoma were treated by ER and the residual Barrett's epithelium was RFA-ablated afterward [21]. The complete remission rates for neoplasia and intestinal metaplasia were 98 and 93 %, respectively. Those excellent results of ER combined with RFA led to the recommendation in most current guidelines that this is the treatment of choice in patients with HGIN and early Barrett's adenocarcinoma [1–3].

Cryoablation

Endoscopic spray cryotherapy either with liquid nitrogen or rapidly expanding carbon dioxide gas leads to a tissue ablation by cooling down the target mucosa [22–24]. So far there is limited data on treatment of early Barrett's neoplasia. However, complete eradication of dysplasia was observed in 87–96 % and complete ablation of Barrett's mucosa in 57–96 % of patients. Cryotherapy was also effective in patients with early Barrett's adenocarcinoma with complete remission in 75 % including patients who had failed other endoscopic treatments.

Cryotherapy remains an experimental treatment until it has demonstrated its efficacy and safety in larger studies with longer follow-up. In addition, this method also has the problem that the target tissue is destroyed making a histological staging of the lesion impossible. Therefore, cryotherapy—like all other ablation methods—should not be used as a primary treatment of Barrett's neoplasia.

Argon-Plasma-Coagulation

Argon plasma coagulation (APC) is a noncontact thermocoagulation procedure with the advantage of a limited depth of penetration—minimizing the risk of perforation. In comparison with RFA or PDT, APC is substantially less expensive. In most cases, partial or complete remission of Barrett's epithelium can be achieved [25–30]. A recently published prospective randomized trial demonstrated that ablation of the remaining nonneoplastic Barrett's esophagus after ER of mucosal Barrett's adenocarcinoma can significantly reduce the rate of recurrences or metachronous neoplasia compared to PPI treatment and follow-up alone [29] (Fig. 6.3).

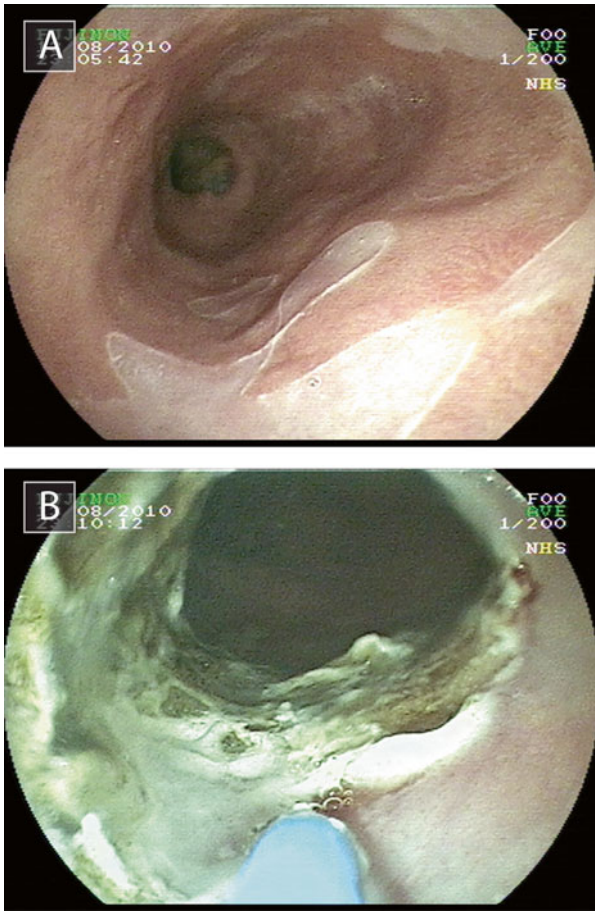

Fig. 6.3 (**a**, **b**) APC treatment of a long-segment Barrett's esophagus after focal ER of a visible lesion with HGIN

The disadvantages of this procedure are the large number of sessions required to achieve complete ablation of Barrett's epithelium and the relatively high risk of residual islands of metaplasia. In addition, it is possible for Barrett's mucosa with a neoplastic potential to remain underneath the neoepithelium and develop to cancer.

Special Indications for Endoscopic Treatment

Low-Grade Intraepithelial Neoplasia

Two recently published studies have shown that low-grade intraepithelial neoplasia (LGIN) is frequently overdiagnosed by nonexpert pathologists [31, 32]. Therefore, most current guidelines recommend obtaining a 2nd opinion by an experienced pathologist in case of a diagnosis of LGIN in Barrett's esophagus [1–3]. However,

when the diagnosis is confirmed, patients with LGIN show a high progression rate to HGIN and adenocarcinoma of up to 13.4 % per year. The high risk for development of more advanced neoplasia raises the question whether an ablative treatment should be performed in patients with confirmed LGIN. This was investigated in a prospective randomized European multicentre trial with 136 patients with confirmed LGIN [33]. Patients were randomized into a treatment arm where ablation of the Barrett's segment with RFA was performed and an observation arm where patients were followed. RFA was able to reduce the progression risk for HGIN and adenocarcinoma by 25 %. These results suggest that patients with bona fide LGIN do have a considerable risk of malignant progression. RFA should therefore be discussed as an alternative to follow-up endoscopies.

Submucosal Barrett's Adenocarcinoma

Submucosal invasion is associated with an increased risk of concurrent lymph node metastasis of up to 41 %. The risk seems to be correlated with the depth of infiltration of the tumor into the submucosal tissue. Adenocarcinoma infiltrating the upper third of the submucosa (pT1sm1; invasion depth up to 500 μm) has a risk varying between 0 and 21 % depending on the presence of further risk factors. Cancer invading the mid and lower part of the submucosa (pT1sm2/3) has concurrent lymph node metastasis in 36–54 % of patients [34–42]. Besides the infiltration depth, further risk factors are poor differentiation grade (G3) and lymphatic (L+) and/or vascular invasion (V+) and all these factors should be taken into account when the patient is discussed in the multidisciplinary tumor board meeting.

Some data suggest that ER can safely be performed in so-called low-risk submucosal cancer (sm1-cancer with invasion up to 500 μm, G1/2, L−, V−) [21, 27]. A recently published study by the Wiesbaden group included 67 patients with submucosal Barrett's cancer invading only the upper layer of the submucosa (T1sm1; ≤500 μm) without the presence of other risk factors [42]. In this large series, all patients were treated by ER, but only one patient demonstrated metastatic disease on follow-up. This was detected by EUS 9 months after treatment, resulting in a lymph node metastasis risk of 1.5 %. Importantly, this risk is still below the usual mortality rate of esophagectomy ranging from 0.5 to 3 % in experienced centers [35–38].

In most recent guidelines, endoscopic treatment is considered an alternative to surgery in patients with so-called low-risk T1sm1-Barrett's adenocarcinoma and this should be discussed with the patient [1–3].

Conclusions

Endoscopic therapy is the treatment of choice for early Barrett's neoplasia. In case of confirmed LGIN without any visible lesion, RFA should be considered, since the rate of progression to a higher grade lesion is high. In patients with HGD and

mucosal Barrett's adenocarcinoma, ER is the first line of treatment. It is not only an effective method, but also serves as a diagnostic tool enabling an exact assessment of the tumor infiltration depth and the presence of further risk factors. To avoid tumor recurrence or metachronous neoplasia ER should always be followed by ablative treatment of the remaining Barrett's mucosa. Therefore, successful endoscopic therapy of HGIN and early Barrett's adenocarcinoma is always a two-step procedure. Step 1: ER of all visible lesions; Step 2: ablation of the remaining flat Barrett's esophagus. According to recent data, ER of early Barrett's adenocarcinoma with incipient infiltration of the submucosa up to 500 μm (pT1sm1) without the presence of further risk factors seems to be safe because the risk of lymph node metastasis is below the mortality rate of radical esophageal resection.

References

1. Bennett C, Vakil N, Bergman J, et al. Consensus statements for management of Barrett's dysplasia and early-stage esophageal adenocarcinoma. Based on a Delphi process. Gastroenterology. 2012;143(2):336–46.
2. Koop H, Fuchs KH, Labenz J, Lynen Jansen P, Messmann H, Miehlke S, Schepp W, Wenzl TG. Mitarbeiter der Leitliniengruppe.S2k-Leitlinie: Gastroösophageale Refluxkrankheit unter Federführung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten. Z Gastroenterol. 2014;52(11):1299–346.
3. Fitzgerald RC, di Pietro M, Ragunath K, Ang Y, Kang JY, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, O'Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyratzopoulos G, de Caestecker J, British Society of Gastroenterology. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. Gut. 2014;63(1):7–42.
4. Inoue H, Endo M. A new simplified technique of endoscopic esophageal mucosal resection using a cap-fitted panendoscope. Surg Endosc. 1993;6:264–5.
5. Pech O, May A, Rabenstein T, Ell C. Endoscopic resection of early oesophageal cancer. Gut. 2007;56:1625–34.
6. Pech O, May A, Manner H, et al. Long-term efficacy and safety of endoscopic resection for patients with mucosal adenocarcinoma of the esophagus. Gastroenterology. 2014;146(3):652–60.
7. Höbel S, Dautel P, Baumbach R, Oldhafer KJ, Stang A, Feyerabend B, Yahagi N, Schrader C, Faiss S. Single center experience of endoscopic submucosal dissection (ESD) in early Barrett's adenocarcinoma. Surg Endosc. 2015;29(6):1591–7.
8. Neuhaus H, Terheggen G, Rutz EM, Vieth M, Schumacher B. Endoscopic submucosal dissection plus radiofrequency ablation of neoplastic Barrett's esophagus. Endoscopy. 2012;44(12):1105–13.
9. Probst A, Aust D, Märkl B, Anthuber M, Messmann H. Early esophageal cancer in Europe: endoscopic treatment by endoscopic submucosal dissection. Endoscopy. 2015;47(2):113–21.
10. Gossner L, Stolte M, Sroka R, et al. Photodynamic ablation of high-grade dysplasia and early cancer in Barrett's esophagus by means of 5-aminolevulinic acid. Gastroenterology. 1998;114:448–55.
11. Overholt BF, Panjehpour M, Haydek JM. Photodynamic therapy for Barrett´s esophagus: follow-up in 100 patients. Gastrointest Endosc. 1999;49:1–7.
12. Pech O, Gossner L, May A, et al. Long-term results of photodynamic therapy with 5-aminolevulinic acid for superficial Barrett's cancer and high-grade intraepithelial neoplasia. Gastrointest Endosc. 2005;62:24–30.

13. Overholt BF, Lightdale CJ, Wang KK, Canto MI, Burdick S, Haggitt RC, Bronner MP, Taylor SL, Grace MG, Depot M. Photodynamic therapy with porfimer sodium for ablation of high-grade dysplasia in Barrett's esophagus: international, partially blinded, randomized phase III trial. Gastrointest Endosc. 2005;62(4):488–98.
14. Overholt BF, Wang KK, Burdick JS, Lightdale CJ, Kimmey M, Nava HR, Sivak Jr MV, Nishioka N, Barr H, Marcon N, Pedrosa M, Bronner MP, Grace M, Depot M. Five-year efficacy and safety of photodynamic therapy with Photofrin in Barrett's high-grade dysplasia. Gastrointest Endosc. 2007;66(3):460–8.
15. Bronner MP, Overholt BF, Taylor SL, Haggitt RC, Wang KK, Burdick JS, Lightdale CJ, Kimmey M, Nava HR, Sivak MV, Nishioka N, Barr H, Canto MI, Marcon N, Pedrosa M, Grace M, Depot M. Squamous overgrowth is not a safety concern for photodynamic therapy for Barrett's esophagus with high-grade dysplasia. Gastroenterology. 2009;136(1):56–64.
16. Yachimski P, Puricelli WP, Nishioka NS. Patient predictors of esophageal stricture development after photodynamic therapy. Clin Gastroenterol Hepatol. 2008;6(3):302–8.
17. Prasad GA, Wang KK, Buttar NS, Wongkeesong LM, Lutzke LS, Borkenhagen LS. Predictors of stricture formation after photodynamic therapy for high-grade dysplasia in Barrett's esophagus. Gastrointest Endosc. 2007;65(1):60–6.
18. Shaheen NJ, Sharma P, Overholt BF, et al. Radiofrequency ablation in Barrett's esophagus with dysplasia. N Engl J Med. 2009;360(22):2277–88.
19. Gupta M, Iyer PG, Lutzke L, et al. Recurrence of esophageal intestinal metaplasia after endoscopic mucosal resection and radiofrequency ablation of Barrett's esophagus: results from a US Multicenter Consortium. Gastroenterology. 2013;145(1):79–86.
20. Haidry RJ, Dunn JM, Butt MA, et al. Radiofrequency ablation and endoscopic mucosal resection for dysplastic barrett's esophagus and early esophageal adenocarcinoma: outcomes of the UK National Halo RFA Registry. Gastroenterology. 2013;145(1):87–95.
21. Phoa KN, Pouw RE, Bisschops R, Pech O, Ragunath K, Weusten BL, Schumacher B, Rembacken B, Meining A, Messmann H, Schoon EJ, Gossner L, Mannath J, Seldenrijk CA, Visser M, Lerut T, Seewald S, Ten Kate FJ, Ell C, Neuhaus H, Bergman JJ. Multimodality endoscopic eradication for neoplastic Barrett oesophagus: results of an European multicentre study (EURO-II). Gut. 2015. pii: gutjnl-2015-309298. doi: 10.1136/gutjnl-2015-309298. [Epub ahead of print].
22. Shaheen NJ, Greenwald BD, Peery AF, Dumot JA, Nishioka NS, Wolfsen HC, Burdick JS, Abrams JA, Wang KK, Mallat D, Johnston MH, Zfass AM, Smith JO, Barthel JS, Lightdale CJ. Safety and efficacy of endoscopic spray cryotherapy for Barrett's esophagus with high-grade dysplasia. Gastrointest Endosc. 2010;71(4):680–5.
23. Dumot JA, Vargo 2nd JJ, Falk GW, Frey L, Lopez R, Rice TW. An open-label, prospective trial of cryospray ablation for Barrett's esophagus high-grade dysplasia and early esophageal cancer in high-risk patients. Gastrointest Endosc. 2009;70(4):635–44.
24. Gosain S, Mercer K, Twaddell WS, Uradomo L, Greenwald BD. Liquid nitrogen spray cryotherapy in Barrett's esophagus with high-grade dysplasia: long-term results. Gastrointest Endosc. 2013;78(2):260–5.
25. Attwood SE, Lewis CJ, Caplin S, et al. Argon beam plasma coagulation as therapy for high-grade dysplasia in Barrett's esophagus. Clin Gastroenterol Hepatol. 2003;1:258–63.
26. Ragunath K, Krasner N, Raman VS, et al. Endoscopic ablation of dysplastic Barrett's oesophagus comparing argon plasma coagulation and photodynamic therapy: a randomized prospective trial assessing efficacy and cost-effectiveness. Scand J Gastroenterol. 2005;40:750–8.
27. Pech O, Behrens A, May AD, et al. Long-Term results and risk factor analysis for recurrence after curative endoscopic therapy in 349 patients with high-grade intraepithelial neoplasia and mucosal adenocarcinoma in Barrett's oesophagus. Gut. 2008;57(9):1200–6.
28. Manner H, Neugebauer A, Scharpf M, et al. The tissue effect of argon-plasma coagulation with prior submucosal injection (hybrid-APC) versus standard APC: a randomized ex-vivo study. United Eur Gastroenterol J. 2014;2(5):383–90.

29. Manner H, Rabenstein T, Braun K, et al. What should we do with the remainder of the Barrett's segment after endoscopic resection of early Barrett's cancer? Intermediate results of the first prospective-randomized trial on the APC ablation of residual Barrett's mucosa with concomitant esomeprazole therapy versus surveillance without ablation after ER of early Barrett's cancer. Endoscopy. 2014;46(1):6–12.
30. Milashka M, Calomme A, Van Laethem JL, et al. Sixteen-year follow-up of Barrett's esophagus, endoscopically treated with argon plasma coagulation. United Eur Gastroenterol J. 2014;2(5):367–73.
31. Curvers WL, ten Kate FJ, Krishnadath KK, Visser M, Elzer B, Baak LC, Bohmer C, Mallant-Hent RC, van Oijen A, Naber AH, Scholten P, Busch OR, Blaauwgeers HG, Meijer GA, Bergman JJ. Low-grade dysplasia in Barrett's esophagus: overdiagnosed and underestimated. Am J Gastroenterol. 2010;105(7):1523–30.
32. Duits LC, Phoa KN, Curvers WL, Ten Kate FJ, Meijer GA, Seldenrijk CA, Offerhaus GJ, Visser M, Meijer SL, Krishnadath KK, Tijssen JG, Mallant-Hent RC, Bergman JJ. Barrett's oesophagus patients with low-grade dysplasia can be accurately risk-stratified after histological review by an expert pathology panel. Gut. 2015;64(5):700–6.
33. Phoa KN, van Vilsteren FG, Weusten BL, Bisschops R, Schoon EJ, Ragunath K, Fullarton G, Di Pietro M, Ravi N, Visser M, Offerhaus GJ, Seldenrijk CA, Meijer SL, ten Kate FJ, Tijssen JG, Bergman JJ. Radiofrequency ablation vs endoscopic surveillance for patients with Barrett esophagus and low-grade dysplasia: a randomized clinical trial. JAMA. 2014;311(12):1209–17.
34. Buskens CJ, Westerterp M, Lagarde SM, Bergman JJ, ten Kate FJ, van Lanschot JJ. Prediction of appropriateness of local endoscopic treatment for high-grade dysplasia and early adenocarcinoma by EUS and histopathologic features. Gastrointest Endosc. 2004;60:703–10.
35. Stein HJ, Feith M, Bruecher BLDM, Nahrig J, Siewert JR. Early esophageal squamous cell and adenocarcinoma: pattern of lymphatic spread and prognostic factors for long term survival after surgical resection. Ann Surg. 2005;242:566–73.
36. Rice TW, Blackstone EH, Goldblum JR, DeCamp MM, Murthy SC, Falk GW, Ormsby AH, Rybicki LA, Richter JE, Adelstein DJ. Superficial adenocarcinoma of the esophagus. J Thorac Cardiovasc Surg. 2001;122:1077–90.
37. Oh DS, Hagen JA, Chandrasoma PT, Dunst CM, Demeester SR, Alavi M, Bremner CG, Lipham J, Rizzetto C, Cote R, Demeester TR. Clinical biology and surgical therapy of intramucosal adenocarcinoma of the esophagus. J Am Coll Surg. 2006;203:152–61.
38. Bollschweiler E, Baldus SE, Schröder W, Prenzel K, Gutschow C, Schneider PM, Hölscher AH. High rate of lymph-node metastasis in submucosal esophageal squamous-cell carcinomas and adenocarcinomas. Endoscopy. 2006;38:149–56.
39. Ancona E, Rampado S, Cassaro M, Battaglia G, Ruol A, Castoro C, Portale G, Cavallin F, Rugge M. Prediction of lymph node status in superficial esophageal carcinoma. Ann Surg Oncol. 2008;15(11):3278–88.
40. Manner H, May A, Pech O, Gossner L, Rabenstein T, Günter E, Vieth M, Stolte M, Ell C. Early Barrett's carcinoma with "low-risk" submucosal invasion: long-term results of endoscopic resection with a curative intent. Am J Gastroenterol. 2008;103(10):2589–97.
41. Badreddine RJ, Prasad GA, Lewis JT, Lutzke LS, Borkenhagen LS, Dunagan KT, Wang KK. Depth of submucosal invasion does not predict lymph node metastasis and survival of patients with esophageal carcinoma. Clin Gastroenterol Hepatol. 2010;8(3):248–53.
42. Manner H, Pech O, Heldmann Y, May A, Pohl J, Behrens A, Gossner L, Stolte M, Vieth M, Ell C. Efficiacy, safety and long-term results of endoscopic treatment for early-stage adenocarcinoma oft he esophagus with low-risk sm1 invasion. Clin Gastroenterol Hepatol. 2013;11(6):630–5.

Chapter 7
Definition, Derivation, and Diagnosis of Barrett's Esophagus: Pathological Perspectives

H. Lowes, T. Somarathna, and Neil A. Shepherd

Introduction

Barrett's esophagus or columnar-lined esophagus (CLO) is defined as the presence of metaplastic columnar or glandular epithelium, where squamous epithelium would be expected, in the distal esophagus, as a result of reflux of gastric and small intestinal contents into the esophagus. Despite drawing global research attention, a precise, evidence-based description of the pathological processes involved in the pathogenesis of CLO has not been achieved and this remains a major research target. Indeed, there is much divergence among research experts regarding etiopathogenetic theories. Nevertheless, the research that has been undertaken to date has progressed our basic understanding of the pathogenesis of CLO dramatically. Such research, and technological advancements, have also changed the diagnostic pathologist's role in the diagnosis of CLO [1].

Notwithstanding uncertainties and disagreements concerning the etiology and pathogenesis, confusion regarding lexicology continues to bedevil Norman Barrett's original characterization of this entity in 1950 [2, 3]. Indeed, Barrett's esophagus is known variously as CELLO (columnar epithelium-lined lower esophagus; Europe), BE (Barrett's esophagus; USA), EBO (endobrachyoesophage; France), and CLO (columnar-lined esophagus; UK, a term principally devised to avoid use of the unfortunate abbreviation of the anglicized spelling of Barrett's esophagus) [1]. The term CLO also embraces short segment CLO, which is the appellation given to

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columnar epithelium extending less than 3 cm above the gastroesophageal junction. Further, so-called ultra-short segment CLO is somewhat of a misnomer, as will be discussed, as this is essentially a gastric disease.

Approaches to both the diagnosis and management of CLO have progressed through various developments in the decades following its original description. Increased recognition of the disease brought a greatly increased workload for pathologists. The pathological workload has the potential to reduce, in response to increasing validity of endoscopic evaluation and the evolution in endoscopic technology. So, the sophisticated endoscopic techniques used in current practice have the potential to largely subsume the pathologist's role in providing the initial diagnosis of CLO. Where biopsies are taken with the suspicion of dysplasia or early malignancy, directed biopsies and EMRs can be taken, rather than the previous laborious Seattle-type biopsy protocols. In addition to reducing expensive histopathological examination, endoscopic advances have obvious clinical benefits. Endoscopic mucosal resections are now widely undertaken in the management of dysplasia and early malignancy, avoiding the extensive morbidity associated with radical surgery [4].

There is an increasing prevalence and incidence of CLO among patients with gastroesophageal reflux disease (GORD) [5–8]. CLO shares many risk factors with GORD (increasing age, abdominal obesity, male sex, white ethnicity, etc. [9–12]) and, indeed, reflux disease itself is an independent risk factor for CLO. There is both a temporal association and an association with GORD symptom severity, with the finding of CLO at endoscopy [13]. Not unexpectedly, the rising incidence of GORD in Western populations is accompanied by an increase in the incidence of CLO, specifically beyond that expected of increasing recognition of the phenomenon, including short segment CLO, and the rate of upper gastrointestinal endoscopy [14, 15]. Historically, cigarette smoking and alcohol consumption have been associated with CLO, although more recent research has failed to confirm these as independent risk factors [16, 17].

The key motivation in identifying CLO is due to a well-mapped potential evolution of gastroesophageal reflux disease to esophageal adenocarcinoma via CLO and glandular dysplasia. A greater risk of adenocarcinoma with a longer length of CLO has been demonstrated [18]. Further, those patients with glandular dysplasia are obviously at a greater risk of developing adenocarcinoma. Thus, through the combined use of endoscopic and histological evaluation, in the future hopefully aided by useful biomarker assessment, it is possible to select a subset of patients who are at greater risk of developing cancer and monitor/survey this cohort accordingly [19].

The Definition of CLO

The recognition of CLO as a premalignant entity gave fresh importance to the development of a precise definition of the disease, in order to accurately delineate an "at risk" patient population. Despite this, the incomplete nature of our understanding of CLO is immediately evident upon comparison of the definitions used between countries. Further initial definitions of Barrett's esophagus used

arbitrary criteria, promoting its designation by a particular length of columnar mucosa, usually cm, extending above the gastroesophageal junction. This was introduced after recognition that the squamo-columnar junction could vary physiologically and could lie up to 2 cm from the true anatomical esophago-gastric junction. Thus, such a criterion attempted to avoid classifying those cases with physiological columnar mucosa at the distal esophagus as CLO [20–22]. However, it was recognized that there was still a risk of adenocarcinoma for pathological CLO segments that extended less than 3 cm above the anatomical esophago-gastric junction, and for this reason, the terminology "short segment Barrett's esophagus" was introduced [22].

Intestinal metaplasia in Barrett's esophagus was first observed in the 1950s but it was Paull and colleagues who championed the three predominant histological phenotypes of CLO, including intestinal metaplasia, in 1976 [23]. Through the 1980s and 1990s, the presence and identification of goblet cells/intestinal metaplasia became enshrined in the definition of CLO, as it was recognized that this phenotype was strongly associated with the development of adenocarcinoma and thus the histological demonstration of intestinal metaplasia and/or goblet cells became the defining feature around the world [24]. This was largely because adenocarcinoma arising in the lower esophagus was "invariably" associated with intestinal metaplasia in the adjacent vicinity and the risk of adenocarcinoma without intestinal metaplasia was purported to be "vanishingly" rare [22]. It is our understanding that this was driven largely by US physicians, with little or no involvement of pathologists, and that there was a failure, at the time, to recognize the fallacies associated with the demonstration of IM pathologically (see later) and the difficulties of differentiating, histologically or by any other means, other conditions at or close to the lower esophagus that might feature goblet cells, most notably intestinal metaplasia in the gastric cardia.

Flejou detailed the marked discrepancies in national diagnostic criteria between different countries in 2007 [25]. In the US, France, Germany, and the Netherlands, intestinal metaplasia remained as the defining diagnostic feature of CLO. In the UK and Japan, the requirement for IM had been dropped (in the UK, by national guidelines published in 2005; see later). However, certainly in the US and Germany, IM remains as the defining feature of CLO to this day. Further the term specialized intestinal metaplasia is still used [24, 26–28], despite clear evidence, now, that there is nothing "specialized" about IM in CLO compared to IM at any other site, especially, of course, in the stomach. The US viewpoint is that, although there are data to suggest that cardia-type epithelium is also premalignant, the importance of IM in the definition of CLO lies in clinical relevance [27]. They argue that there is established premalignant potential appreciable in mucosa that exhibits intestinal metaplasia. While new data do raise the possibility of adenocarcinoma arising in cardia-type mucosa, they maintain that there is insufficient evidence to define the precise risk associated with this phenotype. This is particularly of relevance in a private healthcare system, where the label of a "premalignant condition" has significant economic implications to the patient.

Interestingly, Westerhoff et al have suggested that decreasing the requirement for goblet cells, in the diagnosis of CLO, could increase the diagnosis of Barrett's esophagus by up to 147 % [29]. They have suggested that, as follow-up endoscopy in short columnar segments does not often show goblet cells, the columnar mucosa seen could just represent proximal stomach, and these patients would be erroneously labeled as CLO, should the requirement for goblet cells be removed. It could be argued that such findings merely emphasize the importance of endoscopic correlation, given the prevalence of CLO at endoscopy (0.5–2 %) and USSCLO (CIM; 15–32 %) reported in the West. Westerhoff and colleagues did acknowledge that a part of the argument to include goblet cells in the CLO definition was financially driven.

In support of the US argument, Spechler has maintained that, on his review of the evidence to date, goblet cells should be seen in order to diagnose Barrett's esophagus, citing recent research by Pereira and Chaves to support Westerhoff's conclusions, in which patients with nongoblet cell CLO did not go on to develop IM in subsequent biopsies [24]. The German viewpoint is less dogmatic. Although current German national guidelines do require histopathological evidence of IM, they acknowledge contemporaneously that there is ongoing discussion regarding to what extent columnar epithelium without goblet cells can be diagnosed as CLO [30].

The current US guidelines are in stark contrast to the current national British and Japanese positions, whereby it is widely accepted that intestinal metaplasia, although often seen, is not required for the diagnosis of Barrett's esophagus [31, 32]. Such conflicting opinions are held for a variety of reasons. Scrutiny of the evidence for the implications of IM in the diagnosis of CLO casts significant doubt on the conclusions drawn. Harrison and colleagues have shown that there is a linear relationship between the number of biopsies taken and the demonstration of IM in classical CLO [33]. This was reflected in later work in our own UK Barrett's Oesophagus Registry (UKBOR) study. In our study, if just one biopsy from classical CLO is taken, just over 50 % of cases would show IM whereas, if nine are taken, well over 90 % of classical CLO cases will demonstrate intestinal metaplasia (Fig. 7.1a).

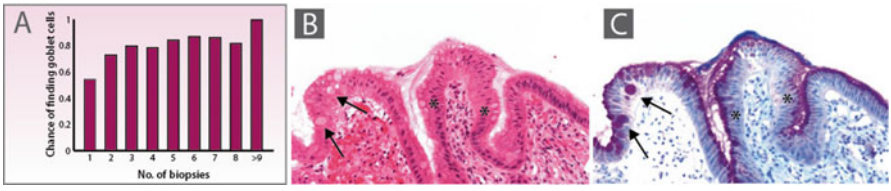

Fig. 7.1 (**a**) Histogram showing data from an unpublished UKBOR (UK Barrett's Oesophagus Registry) study, by Dr T Mandalia and Prof N A Shepherd, of the correlation of the demonstration of goblet cells with the number of biopsies taken at an index endoscopy for the diagnosis of CLO. (**b**, **c**) Pseudo-goblet cells in CLO. Figure (**b**) shows the H & E appearances. The bloated cells (best seen at left and indicated by *arrows*) could easily be passed as goblet cells. However (**c**) shows an ABPAS preparation and this shows PAS-positive neutral mucin in the cells concerned (*arrows*), indicating that they are bloated gastric-type foveolar cells. Background foveolar cells (showing small apical PAS-positive neutral mucin droplets) are indicated by asterisks for comparison

A similar and major potential confounding factor for the importance or otherwise of IM demonstration lies in the method of pathological evaluation of biopsy material. It has been demonstrated that the number of biopsies showing intestinal metaplasia increases with the number of levels at which that biopsy is examined [34]. Many leading British GI pathologists believe that the great majority of long segment CLO cases will show intestinalization somewhere and that not finding it merely represents the frustrating yet inevitable result of sampling bias. Further, there is an embarrassingly high interrater variability, even among leading gastrointestinal pathologists, in the detection of IM. A recent study found that the demonstration of IM, thereby "confirming" the diagnosis of Barrett's esophagus, among such experts, had a kappa value of 0.15 and the presence of goblet cells a kappa value of 0.35 [35]. This study underlines the devastatingly poor interobserver agreement levels, even among expert pathologists, on what actually constitutes intestinal metaplasia/goblet cells, among pathologists, and completely denigrates the utility of IM in defining and diagnosing CLO. An especial difficulty in this regard is differentiating true goblet cells from so-called pseudo-goblet cells, which are bloated foveolar cells with a rounded intracellular mucin content, mimicking true goblet cells (Fig. 7.1b, c). Arguably the subtleties of histopathological assessment of biopsy material have been somewhat overlooked among a research body dominated by clinicians [36].

There is not only uncertainty regarding the quality of evidence supporting the importance of IM, given such sampling bias and poor interrater agreement. Recent research suggests that adenocarcinoma may arise from CLO that does not show intestinal metaplasia. Japanese, German, and British pathologists have shown that adenocarcinoma can arise from cardia-type mucosa in Barrett's esophagus, particularly minute adenocarcinomas [37, 38]. Support is lent to this standpoint by the reported "marching front" of goblet cells, discussed later in this chapter, whereby intestinal-type mucosa is seen more proximally within the Barrett's segment [39]. If it were only intestinal-type epithelium that gave rise to adenocarcinoma, one would expect a greater incidence of adenocarcinoma in the proximal aspect of CLO, which is not observed [40].

These arguments culminated in a seminal paper by two of the world's top gastrointestinal pathologists, both based in North America, who have argued that the demonstration of IM/goblet cells should no longer be required and cited no less than nine reasons why [41]. Some of these are addressed elsewhere in this treatise but they also indicated that goblet cells are uncommon in pediatric patients with CLO, that goblet cells have been shown to wax and wane over the natural history of CLO, and also, importantly, that background, nongoblet cell epithelium has been shown to be biologically intestinalized (vide infra) [41, 42]. Other investigators even report that the presence of goblet cells confers protection against the development of adenocarcinoma [43].

With such a body of evidence indicating that the enthusiastic assimilation of intestinal metaplasia into a defining feature of CLO was somewhat premature, many UK gastrointestinal pathologists have been dissatisfied with specific pathological aspects of the most recent BSG guidelines, published in 2014 [44]. These define

Barrett's esophagus as the replacement of the normal distal esophagus squamous epithelial lining by metaplastic columnar epithelium [44]. The guidelines state that this should both be visible endoscopically and identified to be lying at least 1 cm above the gastroesophageal junction. It includes a vague criterion of "histopathological confirmation," although little detail is given regarding precise histopathological features. This fundamentally shifts the pathological reporting ethos in Barrett's esophagus diagnosis. The 2005 guidelines asserted that the role of the pathologist lay in confirming an endoscopic diagnosis of CLO, with an emphasis on pathological evaluation as critical in complex cases and cases with suspected dysplasia. The more recent guidelines advocate a more independent pathological opinion, a change in practice that we find regressive and unhelpful.

Further, the presence of intestinal metaplasia was reintroduced as a defining feature in the diagnosis of CLO in these BSG guidelines. This was accompanied by a critique of the evidence questioning the importance of IM. While previous authors have raised sampling bias as a potential confounding factor in the investigation of intestinal metaplasia as the heralding predysplastic event in CLO, the most recent guidelines advocate extensive sampling to fully evaluate CLO. Promoting more biopsies to avoid sampling bias in detecting IM is a somewhat flawed argument, given that the evidence to support the need for IM is, in the first place, compromised by sampling bias.

The new BSG guidelines have endorsed the histological finding of "multilayered epithelium" as pathognomonic of CLO [45]. It is our contention that many of the pathological features, purported to be pathognomonic of CLO and thus defining features, may be seen in other situations, namely, in the gastric mucosa or at the esophago-gastric junction, and that the only histological finding capable of independently providing a diagnosis of CLO is that of columnar mucosa immediately juxtaposed with native esophageal structures [1]. It is our view that the diagnosis should not be reliant on the demonstration of IM, not because it isn't important, but because many cases of CLO will be excluded if biopsy numbers are low. Further, relying on the histological demonstration of goblet cells, even without taking into consideration the inaccuracy with which this is done, creates a significant risk of an inappropriate diagnosis of CLO when the diagnosis is really cardia IM. Such a risk is only increased when the importance of endoscopic correlation is actively discouraged.

The Derivation of CLO

Barrett initially described CLO as a segment of tubular, intrathoracic stomach, due to "congenitally short esophagus." This concept was, fortunately, usurped reasonably swiftly by investigators who asserted that CLO was a metaplastic pathological process in the native esophagus, based on a variety of observations, including that the tubular structure macroscopically bears a resemblance to the normal esophagus,

with no peritoneal covering, and, microscopically, that the segment contains submucosal glands and squamous islands [46].

Johns described replacement of the embryonic ciliated columnar epithelium lining the esophagus by squamous epithelium, starting at its mid-point, at the 17th week of development [47]. Remnants of such epithelium were described in both infants and older children [48]. A second congenital theory posited that the columnar epithelium represented a remnant of this embryonic epithelium [49]. Barrett later renounced this theory, noting that, if this were the case, one would expect to see the columnar mucosa arising, at least on occasion, throughout the length of the esophagus, rather than exclusively in the distal portion [50]. Further, congenital remnants of columnar mucosa are more common in the cervical esophagus [51, 52].

Moersch and colleagues were among the first investigators to propose CLO as an acquired phenomenon secondary to reflux esophagitis [53]. An acquired origin of CLO was supported by later studies, citing the acquisition of CLO following surgery known to induce GORD, such as Heller's myotomy [54–57]. Although some claimed reversion of CLO to squamous epithelium after antireflux surgery [58, 59], others attested that it did not "revert" [60]. Later research described the specific importance of chronic injury to the esophageal squamous epithelium. Surgical injury to squamous epithelium in dogs without GORD healed by regeneration of the squamous epithelium [61]. In contrast, animal models, in which surgical techniques were employed to induce reflux disease, provided empirical evidence that CLO was an acquired disease process secondary to GORD and showed that it was a true metaplasia in the esophagus rather than proximal migration of gastric mucosa into the esophagus [61–63]. Other factors have been linked to the development of CLO, including bile reflux, caustic injury by lye, cytotoxic chemotherapeutic agents, gastric acid hypersecretion, and abnormal esophageal motility [64–67]. All share a common outcome of chronic lower esophageal mucosal insult.

Current Theories of the Pathogenesis of CLO

While much of the historical research has focused on the principle that CLO arises as a metaplasia from squamous epithelium following chronic esophageal injury, there are in fact few firm data to support this theory. Indeed, the precise mechanism and genetic changes responsible for the initiation of CLO are still largely unknown. Three main points of contention exist in the theories of derivation of CLO. The first is whether or not the entity arises as a result of clonal expansion of a stem cell niche [68], or whether the columnar epithelium is present due to a postmitotic epigenetic effect inducing transdifferentiation, secondary to gastric refluxate [69]. A second contentious point is that, if CLO arises as a result of stem cell clonal expansion, where do the stem cells originate? Third, a primary concern and interest in CLO is because it is recognized as a premalignant condition. Both historical and more recent guidelines have focused on the intestinal phenotype epithelium as the

significant neoplastic initiator whereas we now recognize the neoplastic potential of epithelium lacking goblet cells as having important neoplastic potential.

CLO Derivation: Stem Cells and Clonal Expansion

There is plentiful evidence to support the theory that CLO arises as a result of proliferation of a stem cell niche rather than metaplasia of native mature squamous mucosa [68]. Research using clonal markers (mitochondrial DNA mutations causing cytochrome c oxidase deficiency) has demonstrated that CLO glands are clonal units, despite exhibiting a variety of cell lineages [68]. This supports the hypothesis that the classical patchwork of columnar phenotypes seen in CLO is actually derived from a small number, if not a single, stem cell.

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) has been shown to be an effective marker of intestinal stem cells in both animal subjects and humans [70–72]. There is LGR5 mRNA expression at the junction of the two different mucosal types seen in the archetypal CLO gland, namely, intestinal epithelium above and basal cardiac-type glands [68]. Immunohistochemistry using Ki-67 shows maximal staining at this point, corroborating this as the likely location of a stem cell niche in the established CLO gland, regardless of where such stem cells originally arose [68, 73].

This classical CLO gland resembles an antral/pyloric gland in its segmental expression of columnar cell types [39]. Toward the luminal aspect, there is intestinal-type epithelium with goblet cells. These cells contain sialomucin and neutral mucin, like normal gastric foveolar cells (expressing MUC 5AC, the trefoil peptide TFF1, MUC 2, and TFF3). The deepest third of the gland aspect contains mucus-secreting cells (expressing MUC 6 and the trefoil peptide TFF2).

Importance has been placed on the development of an intestinal phenotype in CLO as a heralding event in the life course of CLO, traditionally recognized as the "premalignant" step on the pathway to dysplasia [74, 75]. A more recent large epidemiological study supports an association between intestinal metaplasia and the risk of malignancy [76] and was the only recent research cited in the rationale for reintroducing intestinal metaplasia as a necessary diagnostic criterion in the recent BSG guidelines [44]. In our opinion, IM is a late-stage occurrence in the overall disease process and earlier changes can be identified.

It has now been shown that, initially, CLO glands express gastric pyloric-type lineages, with exclusive TFF1+/MUC5AC+ and TFF2+/MUC6+ expression. Neutral drift, random molecular evolution via allele mutations that confer neither advantage nor disadvantage, is thought to be the initial process behind the expression of the various columnar phenotypes that are subsequently observed [39]. Neutral drift is followed by monoclonal expansion of various phenotypes, a process that has been demonstrated using cytochrome C oxidase deficiency as a marker of clonality [77]. In some circumstances, a bias toward an eventual intestinal phenotype is thought to occur due to stem cell niche succession [39]. It is thought that the

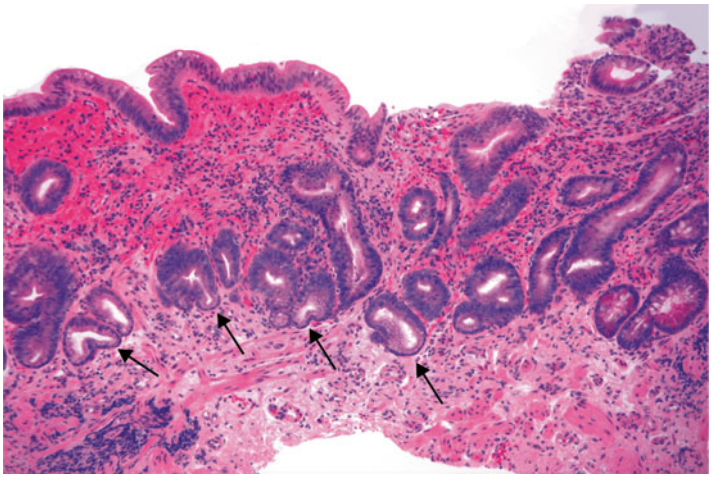

Fig. 7.2 Classical CLO. The superficial glands are all intestinal in type but toward the left and centrally there are gastric-type glands below (*arrows*). These are, in three-dimensional reconstruction studies, in direct communication with the intestinalized glands above. For comparison to the right are intestinal glands with complete IM (*asterisk*)

intestinal phenotype does confer some survival advantage over the gastric phenotype. This microenvironment may itself have been altered by the different phenotypes present [39, 78]. Once monoclonal conversion of the crypt to an intestinal phenotype has occurred, it is likely that the phenotype is fixed at this point. [79] For instance, it is notable that mature intestinalized epithelium, expressing markers of such maturity such as Paneth cells, lacks basal cardiac-type glands in fully established CLO (Fig. 7.2).

Once established, it is clear that CLO evolves along complex pathways in which inflammation likely drives proliferation-induced mutations and epigenetic changes that underpin the observed clonal selection. Esophageal adenocarcinoma arises from this epithelium as the result of stereotypic genetic changes that present morphologically as dysplasia, high-grade dysplasia and, finally, invasive cancer, all through a process involving clonal evolution [80].

Maintenance of Multilineage CLO

The mechanisms for the maintenance of CLO epithelium remain uncertain. In the stomach, the population of gastric glands is thought to be maintained by crypt fission at the stem cell zone, in the neck of the gland [77]. A similar process could drive the maintenance and proliferation of CLO. The patchwork of phenotypes seen could represent multiple, individual monoclonal conversions of individual CLO glands. There is variation in the research data regarding the timeline of the

appearance of an intestinal phenotype but most workers agree that the initial mucosal phenotype arising from stem cells engrafting ulcerated squamous epithelium is nongoblet cell columnar epithelium [81–85]. This is then, variably, followed by the appearance of more specialized CLO phenotypes. However, such phenotypes do not extensively replace the initial cardiac-type epithelium. Patches of nongoblet cell columnar epithelium almost invariably accompany metaplastic intestinal CLO mucosa. Thus, although, certainly initially, the microenvironment favors expansion of nongoblet cell columnar epithelium, the subsequent development of intestinal metaplastic CLO epithelium is not so dominant as to completely replace the historical phenotype.

It has been surmised that the different gland types observed are under selection, either based on their ability to survive caustic injury or on their ability to replicate by fission [39]. CLO has been shown to actively secrete bicarbonate, anions, and claudins, which protect the mucosa from luminal acid, and, of course, mucus, itself highly protective against caustic luminal contents [86–88]. The local microenvironment may favor a certain phenotypic clone in one gland but another in the adjacent.

The Origin of the Initial Stem Cell in CLO

The origin of an initial stem cell unit in CLO remains an enigma. Four mechanistic theories are held in current opinion:

1. Migration of bone marrow stem cells.
2. Stem cells within submucosal ducts or glands regenerating the surface epithelium [89].
3. Stem cell migration from likely metaplastic gastric cardia to repopulate damaged esophageal mucosa [68].
4. Reflux-mediated reprogramming of a resident squamous stem cell [90].

A recent study has showed that bone marrow stem cells can migrate to and engraft the esophagus in a rat model of GORD, using irradiation and esophagojejunostomy [91]. This is in line with earlier research demonstrating the migration of bone marrow stem cells and their subsequent differentiation into squamous cells in esophagitic mucosa [92]. Although this has led to the suggestion that bone marrow stem cells could represent the stem cell of CLO, later research, using surgically induced GORD also in a mouse model, showed that labeled bone marrow progenitor cells did not engraft the GORD-damaged region of the esophagus [93]. As such, the ability of such cells to reconstitute damaged areas of the esophagus in response to GORD-induced damage has not been convincingly demonstrated.

Squamous islands are well recognized in CLO mucosa and are more common after treatment with PPIs (Fig. 7.3a). Using three-dimensional reconstruction techniques, it has been shown that there is an interrelationship between squamous islands and esophageal gland ducts, demonstrating that squamous islands develop

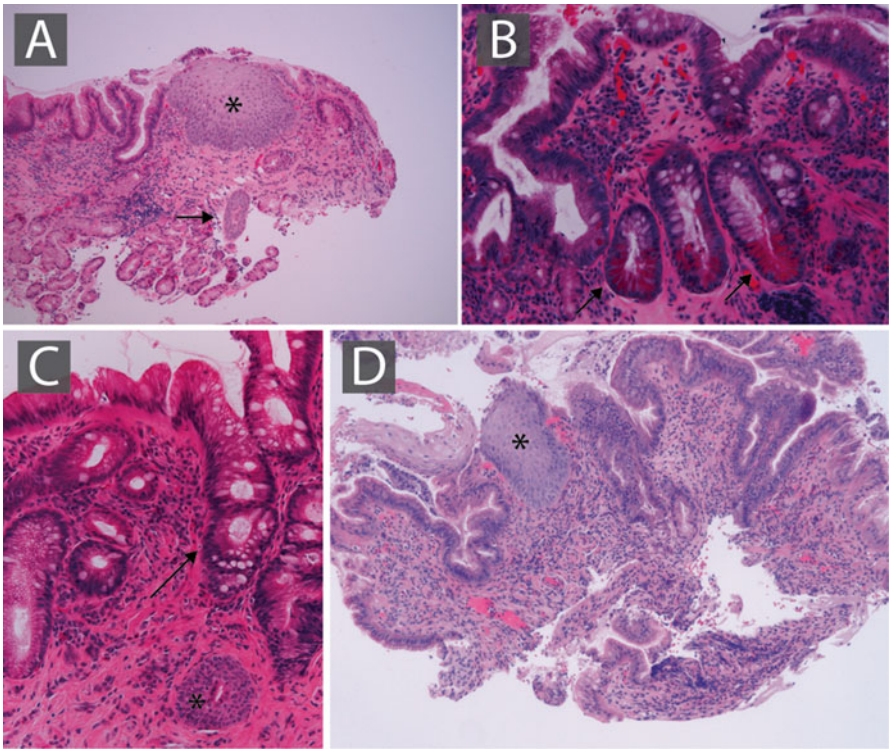

Fig. 7.3 (**a**) A characteristic squamous island in CLO above (*asterisk*) and the esophageal gland duct, from which it arose, below (*arrow*). Such squamous islands are more commonly seen after treatment, promoted by a low acid environment. (**b**) Juxtaposed complete and incomplete intestinal metaplasia in CLO. Note the numerous Paneth cells in the complete IM to the right (*arrows*). (**c**) A native esophageal structure, namely, the esophageal gland duct (*asterisk*), juxtaposed to metaplastic intestinalized glandular mucosa in CLO (*arrow*). This finding is effectively diagnostic of CLO. (**d**) This biopsy appears to show a squamous island (*asterisk*) within glandular mucosa but it is actually taken from the esophago-gastric junction and simply represents the tangentially sectioned squamo-columnar junction. Caution is thus appropriate when diagnosing squamous islands in apparent CLO

as a metaplastic phenomenon from these ducts, likely promoted by a low acid environment [89]. They have also demonstrated an appreciable morphologic continuum between the esophageal gland duct and CLO epithelium, raising the esophageal gland duct as a potential contender for the site of origin of the CLO stem cell [89]. However, it has been argued that the induction of CLO in rat models, which do not have submucosal esophageal glands, renders a stem cell origin at this location less likely [93].

So, more recent theories opine that CLO arises from stem cells located in the gastric cardia and produce glandular epithelium that replaces ulcerated squamous mucosa, due to a local microenvironment that favors proliferation of columnar rather than squamous epithelium [68]. It has also been postulated that the proximal

gastric cardia mucosa, with its relative paucity of parietal and peptic cells, is metaplastic in itself, and that CLO represents a simple extension of this process. Stepwise insults of reflux-associated esophageal squamous injury provide repeated opportunities for the expansion of cardiac mucosal stem cells into the ulcer bed. Columnar epithelium, rather than squamous epithelium, is more suited to the inflamed microenvironment of the distal esophagus in such circumstances. This is termed the cyclic expansion model. It is supported by retrospective studies suggesting that patients with intestinal metaplasia in the cardia progress to develop endoscopically identifiable CLO [39].

There is also support for a basal squamous epithelial stem cell as the potential stem cell initiating CLO. A recent study used CD34 and EpCAM immunostaining to identify progenitor stem cells in three-dimensional epithelial whole mounts of esophageal squamous epithelium [94]. The results suggest that stem cell-like properties, namely, plasticity in self-renewal, were seen in a widespread manner, throughout the depth of the epithelium. They noted that the maximum proliferative activity was seen in the interpapillary basal region of the squamous epithelium and this corroborates other recent studies suggesting this as a potential location of the stem cell origin in CLO [69, 94].

The Derivation of Intestinal Metaplasia

While a "complete" phenotype of intestinal differentiation can be seen in CLO (termed type I intestinal metaplasia and primarily seen in the distal CLO segment, Fig. 7.3b), the more common occurrence is incomplete metaplasia [39]. In the latter, the columnar mucosa does exhibit a focal foveolar phenotype and gastrin mucin core expression can be demonstrated. Further, it has been demonstrated that even those glands exhibiting cardiac or mucinous epithelium only show evidence of intestinal differentiation biologically by way of villin expression (17% of cases) and CDX2 expression (43% of cases) [42, 95, 96]. Such findings highlight the incongruity of using morphological intestinal metaplasia as a specific criterion for the diagnosis of CLO.

A gradient of goblet cell density has been described, whereby goblet cell columnar mucosa is more prevalent in the proximal CLO segment [23, 97, 98]. Indeed, a zonal distribution of the various phenotypes of columnar mucosa seen in CLO has long been described [23]. "Specialized" intestinal metaplastic mucosa, it is said, is seen in the distal segment closely mixed with cardiac and oxyntocardiac mucosa. Proximally, the intestinal mucosal phenotype appears as a uniform front abutting the squamo-columnar mucosa [39]. The development and fixation of an intestinal phenotype may be associated with expression of the homeobox gene CDX2 in CLO glands [99, 100]. In the duodenum, bile salt action induces CDX2 expression [100, 101]. It is known that injurious bile salts are most soluble at an intermediate pH, the environment found proximally in CLO [100, 101]. These findings have given birth to the theory of the "marching front" of intestinalized mucosa in CLO, and corre-

lates with the gradient of pH known to occur in the disease, namely, that the luminal pH decreases distally [39].

It has been shown that gastric phenotype epithelium with CDX2 expression exists in both tumors and the cardiac-type mucosa adjacent to tumors, a finding suggesting that such tumors arise from CDX2 positive cardiac-type epithelium. However, the extent of intestinal metaplasia in CLO with adenocarcinoma is associated with the length of the CLO segment but is independent of tumor size, suggesting that development of the phenotype is an epiphenomenon rather than a preneoplastic condition [38]. It has also been reported that gastric-immunophenotype tumors are strongly correlated with minute tumor size [37]. A key research target is the understanding of what specific properties of the CLO cells render them susceptible to dysplastic progression. It is likely that it is not solely the properties of CLO cells, but also the overall microenvironment, that lead to neoplastic progression. On a more general level, it has been argued that precursors of malignancy most likely depend, not only on genetic changes, but also crucially on competition between clonal cell lineages within the biomechanical structure of the tissue, so that certain clones are able to overcome the native functional integrity of the tissue [102].

The Diagnosis of CLO

Endoscopic Diagnosis

The established potential of neoplasia renders the diagnosis of CLO of utmost importance, whether achieved through endoscopic or histological techniques. The endoscopist should be as certain as is possible that the native esophagus has been biopsied, and indicate as such to the pathologist, in order to permit accurate histological assessment. The definition of the gastroesophageal junction varies between countries. In Japan, it is defined by the lower limit of the palisade longitudinal vessels at endoscopy [40]. This is both a useful endoscopic and histological marker. The location of these vessels is unaffected by the motion of the esophagus with respiratory effort, and air insufflation during the procedure, unlike, potentially, the proximal extent of the gastric rugal folds, as generally used in the UK [103]. These longitudinal vessels in the lower esophagus are also absent in mucosal resections of the stomach, when defined as veins at least 100 μm in caliber, lying above the muscularis mucosae [104].

The endoscopic report in its entirety is required to accompany the histology specimen if a meaningful histological assessment of that specimen is to be carried out. This should include both the endoscopic findings, including the presence of a hiatus hernia, and technical information such as the distance of the biopsy site from the incisors. Diagnosis is certainly achievable by endoscopic findings alone, especially, of course, in long segment CLO and pathology, then,

is, at best, corroborative of the diagnosis. Pathology is more useful when there is endoscopic uncertainty, especially in the presence of stricturing, ulceration, and in short segment disease [1].

Short Segment and Ultra-Short Segment CLO

Short segment CLO is defined as CLO to a length of less than 3 cm. Ultra-short segment CLO (USSCLO) is a pathological diagnosis and, yet, there is still major variation in how this diagnosis is applied. We believe that the latter diagnosis should be restricted to the occurrence of intestinal metaplasia in the gastric cardia. As such, it is a gastric disease and is associated with Helicobacter pylori infection rather than reflux disease [105, 106]. Arguably the term cardia intestinal metaplasia (CIM) is more appropriate. However, some use the term USSCLO to describe CLO that was not identified endoscopically. The importance of accurate endoscopic correlation with pathological interpretation is highlighted by the fact that classical CLO, short segment CLO, and CIM can show identical microscopic features. Of course, dysplasia arises much less frequently in CIM compared with short segment CLO, and much less frequently in short segment CLO compared with classical CLO [31, 34]. Nevertheless, the overall incidence of neoplastic change in these three conditions may well be similar because classical CLO is much rarer (about 1–2 % of a Western population) than SSCLO (8–17 % of a Western population) and USSCLO (15–32 % of a Western population).

Biopsy Protocols

The advent of modern endoscopic techniques, including high resolution endoscopy, autofluorescence imaging, confocal endomicroscopy, and narrow band imaging, has facilitated a move from laborious Seattle protocol surveillance biopsies to targeted surveillance biopsy. Historically, the Seattle-type biopsy protocols, whereby quadrantic biopsies are taken along every 2 cm in the CLO segment, were thought appropriate due to the incongruity of pathological findings with endoscopic appearances. Notably, macroscopically benign mucosa on routine endoscopy had been identified as CLO with high-grade dysplasia and even adenocarcinoma on microscopic analysis [107]. Recently, newer endoscopic techniques, particularly high-resolution endoscopy with acetic acid chromoendoscopy, have been cited as reliably identifying a majority of lesions with high-grade dysplasia and adenocarcinoma. Detection rate in low-risk groups showed no significant differences between high-resolution chromoendoscopy alone and Seattle protocol biopsies and only an additional three cases in a high-risk group were identified [108–110].

Histological Features

It should be emphasized that, in routine practice, endoscopic findings are key in the diagnosis of CLO and that the entity is usually not a pathological diagnosis alone. Indeed, in many cases pathology will play no part in the diagnosis. In a larger proportion the pathology report will only serve to corroborate endoscopic appearances, which in turn should be interpreted along with clinical aspects. However, initially, it is appropriate to acknowledge that, in about 10–15% of classical CLO cases, a definitive diagnosis is possible by pathology. This is when biopsies, usually serendipitously, include native esophageal structures, namely, esophageal submucosal glands and/or their ducts, and these are seen juxtaposed to metaplastic glandular mucosa (Fig. 7.3c) [111]. In this situation, it is evident that the biopsy in question derives from the true native esophagus and that native esophagus shows glandular metaplasia, thus assuring the diagnosis of CLO. However, it is emphasized that this is only seen in a small proportion of cases and is inevitably beholden to the number and size of biopsies taken.

What about isolated squamous islands among columnar mucosa? Squamous islands are certainly seen in untreated CLO but are also seen more commonly in treated CLO, especially with PPIs [112, 113]. Here they are thought to derive from the native esophageal gland ducts [89]. However, apparent squamous islands can also be seen at the gastroesophageal junction, particularly where mucosal biopsies have not been well orientated, and here they actually represent an irregular Z-line and tangential sectioning of the adjacent squamous and glandular mucosa (Fig. 7.3d) [1]. So, squamous islands may be helpful in corroborating a diagnosis of CLO and may indicate the effects of treatment but they are certainly not specific to CLO.

A multilayered, transitional-type epithelium, with features of both squamous and columnar differentiation, is described as a hallmark feature of CLO (Fig. 7.4).

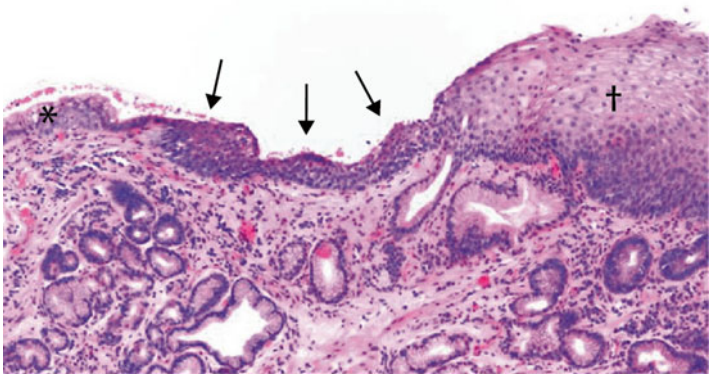

Fig. 7.4 Multilayered epithelium in CLO (*arrows*). It resemblance to immature squamous metaplasia in the uterine cervix is notable here. In this section, the multilayered epithelium is bordered by columnar epithelium on the left (*asterisk*) and by squamous epithelium on the right (*dagger*)

Although this has variably been described as the precursor lesion to CLO, a histological marker of CLO and a histological marker of anatomical site, it is now clear that this lesion is not specific for CLO [45, 114–117]. This epithelium can be seen at the normal esophago-gastric junction, in heterotopic gastric mucosa in the upper esophagus and as a treatment effect following ablative therapy in CLO. In line with its apparent physiological occurrence, it has been shown to be highly prevalent in the Japanese population, where there is a low incidence of CLO [1, 118].

Of very considerable importance now as a symbol of the way CLO intestinalization develops, the so-called hybrid gland was originally thought to be pathognomonic for CLO (Fig. 7.5a) [116]. Hybrid glands show a well-defined cutoff between cardia-type mucinous glands below and superficial intestinal metaplasia above [68]. It may be that the observed transition point represents the stem cell zone in the CLO gland, where the varying phenotypic clones are competing or in the process of monoclonal conversion of the gland. More recently, such glands have since been identified in the stomach, where they represent the transition point between native gastric glands and intestinal metaplasia, both in the gastric antrum and in the cardia [68]. So, while not specific to CLO, it is clear that such hybrid glands are seen much more commonly in CLO than in the native stomach and some have postulated that, in immature intestinal phenotypes, there is transition from gastric-type epithelium, below, to intestinal-type epithelium above, in most CLO glands. So, it is becoming increasingly clear that such glands can be diagnostically useful in the assessment of CLO, although they should not be regarded as pathognomonic.

Cardiac, fundic, intestinal and, on occasion pancreatic, phenotypes of metaplastic epithelium are all seen in CLO (Fig. 7.5b). There has been extensive debate regarding the significance of each phenotype. In North America, so-called special-

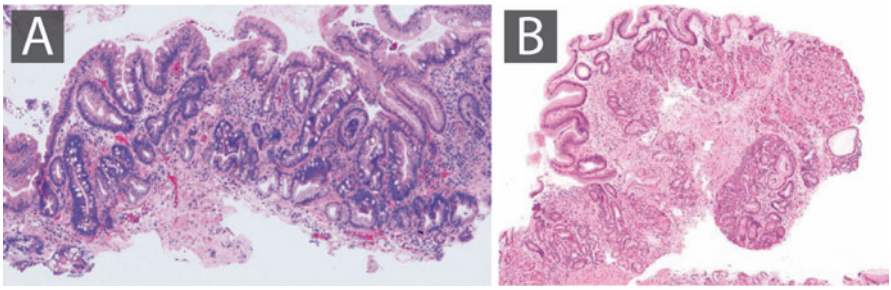

Fig. 7.5 (**a**) So-called hybrid glands in CLO. Here the coexistence of intestinal-type epithelium above and gastric-type epithelium below is well seen, in the same gland, in numerous glands toward the lower aspect of this CLO biopsy. Despite our misgivings about IM as the definitive marker of CLO, this bidirectional differentiation within an individual gland is so prevalent in CLO, and uncommon in the stomach, such that it may be used as a potential marker for the diagnosis of CLO. (**b**) Pancreatic phenotype in CLO. Most of the epithelium is of fundic type but the somewhat darker lobule, lower right, is pancreatic in type

ized intestinal metaplasia remains a requisite for the diagnosis of CLO [119]. This had not been the case in the UK for some time, although the recent BSG guidelines may change the diagnostic landscape in this respect. Regardless of the fact that there is little positive evidence for the risk of neoplasia in CLO to be specifically associated with an intestinal phenotype, there is marked intraobserver variation in the reporting of intestinal metaplasia, even among specialist gastrointestinal pathologists [35]. Intestinal metaplasia also occurs as a skip lesion and as such sampling bias effects results. It is also more likely to occur in more proximal CLO biopsies, as previously discussed [120].

Indeed, as discussed earlier, immunohistochemical studies have shown that metaplastic epithelium, with morphological features of cardiac-type epithelium, biologically express an intestinal phenotype, arguably negating the importance of morphological appearances [37, 95, 121]. For these reasons, it is our opinion that intestinal metaplasia is not critical for the diagnosis of CLO in biopsies taken at the index endoscopy. Similarly, assessment of complete or incomplete metaplasia is regarded as an academic endeavor alone and not necessary in routine CLO biopsy reporting [1].

Finally, CLO is also characterized by dramatic changes in the mucosal musculature. There is hyperplasia and duplication of the muscularis mucosae, for reasons that are as yet unclear (Fig. 7.6a). Further, the lamina propria itself can become muscularized in CLO (Fig. 7.6b) and this suggests that mucosal prolapse may be a pathogenetic factor in the muscle changes that characterize CLO [122–124]. It is now accepted that the deepest aspect of the demonstrable muscularis mucosae represents the original muscularis mucosae and that tumor that has not fully penetrated the muscularis mucosae is not yet in the submucosa. Nevertheless, changes in the muscularis mucosae still cause especial difficulty in staging early adenocarcinoma arising from CLO.

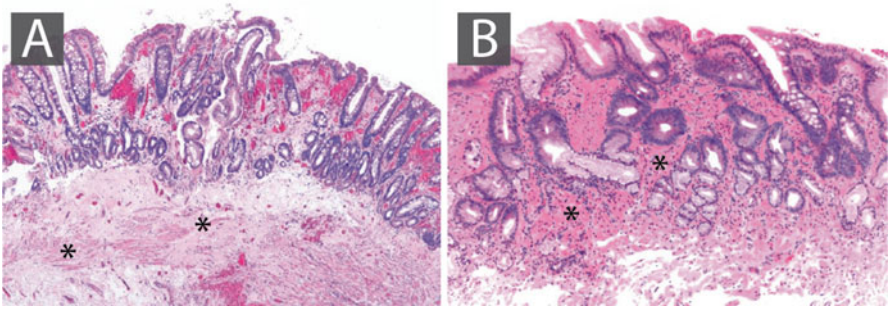

Fig. 7.6 (**a**) Intestinalized CLO mucosa above with the typical splaying and thickening of the muscularis mucosae well seen below (*asterisks*). (**b**) A CLO biopsy. Not only is there the superficial intestinal and deep gastric phenotype to the glands, there is also intense muscularization of the lamina propria, here appearing deep red (*asterisks*). Elsewhere in the gut, this is a pathological feature of mucosal prolapse but it is uncertain whether this feature in CLO represents mucosal prolapse in the esophagus

Gastric Heterotopia

A potential pathological mimic of CLO is gastric heterotopia. However, this occurs in the upper esophagus, seen at endoscopy as a discrete pink and velvety oval-shaped lesion. The term "inlet patch" is given to such heterotopia arising in the subcricoid region. Although this usually generally represents congenital remnants of the fetal esophagus columnar lining, trauma can also induce a similar phenomenon. The incidence of esophageal gastric heterotopia is not well defined. A recent study found it to be present in 1 % of the study population [125]. This is not necessarily representative of the normal population, as 20 % of participants also had CLO. Gastric heterotopia usually exhibits specialized epithelium, potentially causing associated ulceration of the surrounding squamous mucosa. Colonization by helicobacter pylori has also been described [126]. Although intestinal metaplasia and dysplasia is described in such entities, it is exceedingly rare. As always, correlation with endoscopic findings is critical in correctly diagnosing this benign entity and in assuring that it is not confused with its lower esophageal glandular counterpart, CLO.

Hiatus Hernia

The presence of a hiatus hernia, as is commonly seen in association with CLO, may make interpretation of diagnostic biopsies difficult. It is now accepted that classical CLO is almost always associated with a hiatus hernia. Occasionally, biopsies from a hiatus hernia may be submitted in the mistaken belief, by an endoscopist, that they derive from CLO. In this situation, it can be impossible for a pathologist to differentiate the two although the pathologist can have a high degree of suspicion that the biopsy is from native gastric mucosa if there is very well-structured gastric fundic-type epithelium (Fig. 7.7). However, inflammation and intestinal metaplasia may also be present in hiatus hernia mucosa and this may cause profound mimicry of CLO. The combination of endoscopic confusion and/or ignorance and nonspecific pathological changes with intestinalization, once again, exemplifies why the dogmatic reliance on the histopathological demonstration of IM/goblet cells may well lead to an erroneous diagnosis of CLO.

Immunohistochemical Findings

The ability of immunohistochemistry to distinguish native gastric mucosa from metaplastic glandular mucosa in the esophagus has been the subject of intense literature debate. Thus, initially, there was a proposal for a specific intermediate filament CK7/CK20 expression profile of CLO. This was described as luminal,

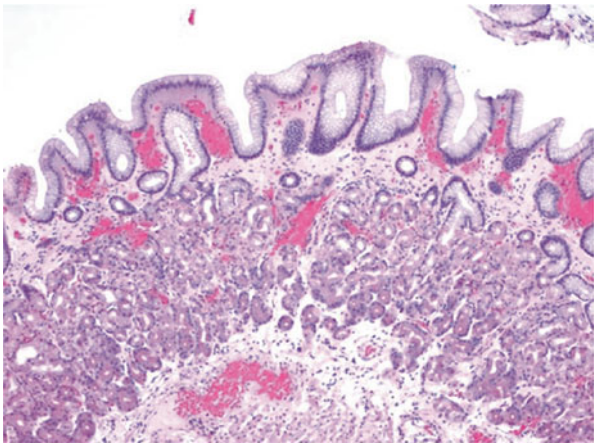

Fig. 7.7 Mature and very well-structured fundic-type epithelium. This can be seen in CLO but its presence should alert the pathologist to consider an alternative diagnosis of hiatus hernia

weak CK20 positivity, with intense CK7 staining at the luminal and deep glands [127–129]. However, many workers, including ourselves, have found this phenotype by no means specific to CLO and have shown an identical staining pattern in incomplete intestinal metaplasia in the stomach. Thus, currently, there is no support for the routine use of CK7 and CK20 immunohistochemistry in the diagnosis of CLO and to distinguish it from cardia IM [1]. More recently, it has been proposed that immunoreactivity for p53 and Ki67 may be of benefit in evaluating dysplasia in CLO [130]. Even in this regard, we are not convinced. We do not believe that any immunohistochemical result will significantly alter a diagnosis made on morphological grounds. If it is uncertain whether or not dysplasia is present, then there is a highly appropriate diagnostic category, namely, mucosa indefinite for dysplasia, and we are not convinced that any immunohistochemical result will change that final diagnostic label.

Conclusions

Our understanding of the etiopathogenesis of CLO has progressed very significantly since its original description more than 50 years ago. It would seem most likely that the disease represents a metaplastic replacement of damaged squamous mucosa by gastric-type glandular mucosa and that continued insults result in intestinalization of those gastric glands to produce the classical "mixed" phenotype of CLO. Further, it is now evident that the diagnosis can be accurately achieved through the use of advanced endoscopic technology and it is likely that endoscopy will ultimately replace pathology completely as the major diagnostic modality. Even so, pathological appraisal of CLO biopsies is likely to continue and the controversies of what features are required to make the diagnosis remain. Pathological assessment continues to rely mainly on morphological appearances and there is

little evidence for the routine use of histochemistry or immunohistochemistry in the pathological assessment of CLO. There are still very considerable controversies regarding the derivation of CLO and we fervently need a better understanding of how CLO develops to be able to more successfully reverse the process and reduce the ever-increasing prevalence of neoplastic complications of CLO.

References

1. Hopcroft SA, Shepherd NA. The changing role of the pathologist in the management of Barrett's oesophagus. Histopathology. 2014;65(4):441–55.
2. Barrett NR. Chronic peptic ulcer of the oesophagus and oesophagitis. Br J Surg. 1950;38:175–82.
3. Spechler SJ. The columnar-lined esophagus. History, terminology, and clinical issues. Gastroenterol Clin North Am. 1997;26(3):455–66.
4. Williamson JM, Almond LM, Shepherd NA, Barr H. Current management of Barrett's oesophagus. Br J Hosp Med (Lond). 2012;73(5):271–7.
5. Winters Jr C, Spurling TJ, Chobanian SJ, Curtis DJ, Esposito RL, Hacker 3rd JF, et al. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. Gastroenterology. 1987;92(1):118–24.
6. Eisen GM, Sandler RS, Murray S, Gottfried M. The relationship between gastroesophageal reflux disease and its complications with Barrett's esophagus. Am J Gastroenterol. 1997;92(1):27–31.
7. Csendes A, Smok G, Burdiles P, Quesada F, Huertas C, Rojas J, et al. Prevalence of Barrett's esophagus by endoscopy and histologic studies: a prospective evaluation of 306 control subjects and 376 patients with symptoms of gastroesophageal reflux. Dis Esophagus. 2000;13(1):5–11.
8. Lieberman DA, Oehlke M, Helfand M. Risk factors for Barrett's esophagus in community-based practice. GORGE consortium. Gastroenterology Outcomes Research Group in endoscopy. Am J Gastroenterol. 1997;92(8):1293–7.
9. Edelstein ZR, Farrow DC, Bronner MP, Rosen SN, Vaughan TL. Central adiposity and risk of Barrett's esophagus. Gastroenterology. 2007;133(2):403–11.
10. Eloubeidi MA, Provenzale D. Clinical and demographic predictors of Barrett's esophagus among patients with gastroesophageal reflux disease: a multivariable analysis in veterans. J Clin Gastroenterol. 2001;33(4):306–9.
11. Corley DA, Kubo A, Levin TR, Block G, Habel L, Rumore G, et al. Race, ethnicity, sex and temporal differences in Barrett's oesophagus diagnosis: a large community-based study, 1994–2006. Gut. 2009;58(2):182–8.
12. El-Serag HB, Kvapil P, Hacken-Bitar J, Kramer JR. Abdominal obesity and the risk of Barrett's esophagus. Am J Gastroenterol. 2005;100(10):2151–6.
13. Singh P, Taylor RH, Colin-Jones DG. Esophageal motor dysfunction and acid exposure in reflux esophagitis are more severe if Barrett's metaplasia is present. Am J Gastroenterol. 1994;89(3):349–56.
14. Prach AT, MacDonald TA, Hopwood DA, Johnston DA. Increasing incidence of Barrett's oesophagus: education, enthusiasm, or epidemiology? Lancet. 1997;350(9082):933.
15. van Soest EM, Dieleman JP, Siersema PD, Sturkenboom MC, Kuipers EJ. Increasing incidence of Barrett's oesophagus in the general population. Gut. 2005;54(8):1062–6.
16. Cook MB, Shaheen NJ, Anderson LA, Giffen C, Chow WH, Vaughan TL, et al. Cigarette smoking increases risk of Barrett's esophagus: an analysis of the Barrett's and Esophageal Adenocarcinoma Consortium. Gastroenterology. 2012;142(4):744–53.

17. Kubo A, Levin TR, Block G, Rumore G, Quesenberry Jr CP, Buffler P, et al. Cigarette smoking and the risk of Barrett's esophagus. Cancer Causes Control. 2009;20(3):303–11.
18. Menke-Pluymers MBE, Hop WCJ, Dees J, van Blankenstein M, Tilanus HW. Risk factors for the development of an adenocarcinoma in columnar-lined (Barrett) esophagus. Cancer. 1993;72(4):1155–8.
19. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011;365(15):1375–83.
20. Bombeck CT, Dillard DH, Nyhus LM. Muscular anatomy of the gastroesophageal junction and role of phrenoesophageal ligament; autopsy study of sphincter mechanism. Ann Surg. 1966;164(4):643–54.
21. Takubo K, Arai T, Sawabe M, Miyashita M, Sasajima K, Iwakiri K, et al. Structures of the normal esophagus and Barrett's esophagus. Esophagus. 2003;1(1):37–47.
22. Riddell RH. The biopsy diagnosis of gastroesophageal reflux disease, "carditis", and Barrett's esophagus, and sequelae of therapy. Am J Surg Pathol. 1996;20 Suppl 1:S31–50.
23. Paull A, Trier JS, Dalton MD, et al. The histologic spectrum of Barrett's esophagus. N Engl J Med. 1976;295:476–80.
24. Spechler SJ. Barrett's esophagus: is the goblet half-empty? Clin Gastroenterol Hepatol. 2012;10(11):1237–8.
25. Flejou JF, Svrcek M. Barrett's oesophagus—a pathologist's view. Histopathology. 2007;50(1):3–14.
26. Sampliner RE. Updated guidelines for the diagnosis, surveillance, and therapy of Barrett's esophagus. Am J Gastroenterol. 2002;97:1888–95.
27. American Gastroenterology Association. AGA medical position statement on the management of Barrett's esophagus. Gastroenterology. 2011;140:1084–91.
28. Wang KK, Sampliner RE. Updated guidelines for Barrett's esophagus. Am J Gastroenterol. 2008;103:788–97.
29. Westerhoff M, Hovan L, Fau-Lee C, Lee C, Fau-Hart J, Hart J. Effects of dropping the requirement for goblet cells from the diagnosis of Barrett's esophagus. Clin Gastroenterol Hepatol. 2012;10(11):1232–6.
30. Koop H, Fuchs KH, Labenz J, Lynen Jansen P, Messmann H, Miehlke S, et al. S2k guideline: gastroesophageal reflux disease guided by the German Society of Gastroenterology: AWMF register no. 021-013. Z Gastroenterol. 2014;52(11):1299–346.
31. Watson AJ, Heading R, Shepherd NA. Guidelines for the diagnosis and management of Barrett's columnar lined oesophagus. A report of the working party of the British Society of Gastroenterology. 2005. http://wwwbsg.org.uk. Accessed 18 May 2015.
32. Aoki T. Report of research committee on definition of Barrett's esophagus (epithelium). Japanese Society of Esophageal Diseases: Chiba; 2000.
33. Harrison R, Perry I, Haddadin W, McDonald S, Bryan R, Abrams K, et al. Detection of intestinal metaplasia in Barrett's esophagus: an observational comparator study suggests the need for a minimum of eight biopsies. Am J Gastroenterol. 2007;102(6):1154–61.
34. Weston AP, Krmpotich P, Makdisi WF, Cherian R, Dixon A, McGregor DH, et al. Short segment Barrett's esophagus: clinical and histological features, associated endoscopic findings, and association with gastric intestinal metaplasia. Am J Gastroenterol. 1996;91(5):981–6.
35. Wang H, Brown I, Kumarasinghe P, Langner C, Lauwers G, Shepherd N, Vieth M, Srivastava A, Odze R. Poor agreement for detection of goblet cells in esophageal and GEJ biopsies. Modern Pathol. 2012;25(Suppl):184–185A.
36. Spechler SJ, Goyal RK. Barrett's esophagus. N Engl J Med. 1986;315:362–71.
37. Takubo K, Aida J, Naomoto Y, et al. Cardiac rather than intestinal-type background in endoscopic resection specimens of minute Barrett adenocarcinoma. Hum Pathol. 2009;40:65–74.
38. Watanabe G, Ajioka Y, Takeuchi M, Annenkov A, Kato T, Watanabe K, Tani Y, Ikegami K, Yokota Y, Fukuda M. Intestinal metaplasia in Barrett's oesophagus may be an epiphenomenon rather than a preneoplastic condition, and CDX2-positive cardiac-type epithelium is associated with minute Barrett's tumour. Histopathology. 2014;66:201–14.

39. McDonald SAC, Graham TA, Lavery DL, Wright NA, Jansen M. The Barrett's gland in phenotype space. Cell Mol Gastroenterol Hepatol. 2015;1:41–54.
40. Takubo K, Vieth M, Aida J, Sawabe M, Kumagai Y, Hoshihara Y, et al. Differences in the definitions used for esophageal and gastric diseases in different countries: endoscopic definition of the esophagogastric junction, the precursor of Barrett's adenocarcinoma, the definition of Barrett's esophagus, and histologic criteria for mucosal adenocarcinoma or high-grade dysplasia. Digestion. 2009;80(4):248–57.
41. Riddell RH, Odze RD. Definition of Barrett's esophagus: time for a rethink--is intestinal metaplasia dead? Am J Gastroenterol. 2009;104(10):2588.
42. Hahn HP, Blount PL, Ayub K, Das KM, Souza R, Spechler S, et al. Intestinal differentiation in metaplastic, non-goblet columnar epithelium in the esophagus. Am J Surg Pathol. 2009;33(7):1006–15.
43. Golden K, Srivastava A, Sanchez CA, et al. High goblet cell density protects against progression to adenocarcinoma in patients with Barrett's esophagus. Mod Pathol. 2011;24:149A.
44. Fitzgerald RC, di Pietro M, Ragunath K, Ang Y, Kang J-Y, Watson P, et al. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. Gut. 2013.
45. Glickman JN, Spechler SJ, Souza RF, Lunsford T, Lee E, Odze RD. Multilayered epithelium in mucosal biopsy specimens from the gastroesophageal junction region is a histologic marker of gastroesophageal reflux disease. Am J Surg Pathol. 2009;33(6):818–25.
46. Allison PR, Johnstone AS. The oesophagus lined with gastric mucous membrane. Thorax. 1953;8(2):87–101.
47. Johns BA. Developmental changes in the oesophageal epithelium in man. J Anat. 1952;86(4):431–42.
48. Rector CM. Aberrant mucosa in the esophagus in infants and in children. Arch Pathol. 1941;31:285–94.
49. Barrett NR. The lower oesophagus lined by columnar epithelium. London: Butterworth & Co.; 1958.
50. Barrett NR. Benign stricture in the lower esophagus. J Thorac Cardiovasc Surg. 1962;43:703–15.
51. Chong VH. Heterotopic gastric mucosal patch of the proximal esophagus. Croatia: InTech Publishing; 2011.
52. von Rahden BH, Stein HJ, Becker K, Liebermann-Meffert D, Siewert JR. Heterotopic gastric mucosa of the esophagus: literature-review and proposal of a clinicopathologic classification. Am J Gastroenterol. 2004;99(3):543–51.
53. Moersch RN, Ellis Jr FH, Mc DJ. Pathologic changes occurring in severe reflux esophagitis. Surg Gynecol Obstet. 1959;108(4):476–84.
54. Kortan P, Warren RE, Gardner J, Ginsberg RJ, Diamant NE. Barrett's esophagus in a patient with surgically tested achalasia. J Clin Gastroenterol. 1981;3(4):357–60.
55. Meyer W, Vollmar F, Bar W. Barrett-esophagus following total gastrectomy. A contribution to it's pathogenesis. Endoscopy. 1979;11(2):121–6.
56. Hamilton SR, Yardley JH. Regnerative of cardiac type mucosa and acquisition of Barrett mucosa after esophagogastrostomy. Gastroenterology. 1977;72(4 Pt 1):669–75.
57. Naef AP, Savary M, Ozzello L. Columnar-lined lower esophagus: an acquired lesion with malignant predisposition. Report on 140 cases of Barrett's esophagus with 12 adenocarcinomas. J Thorac Cardiovasc Surg. 1975;70(5):826–35.
58. Radigan LR, Glover JL, Shipley FE, Shoemaker RE. Barrett esophagus. Arch Surg. 1977;112(4):486–91.
59. Ransom JM, Patel GK, Clift SA, Womble NE, Read RC. Extended and limited types of Barrett's esophagus in the adult. Ann Thorac Surg. 1982;33(1):19–27.
60. Mangla JC. Barrett's esophagus: an old entity rediscovered. J Clin Gastroenterol. 1981;3(4):347–56.
61. Gillen P, Keeling P, Byrne PJ, West AB, Hennessy TP. Experimental columnar metaplasia in the canine oesophagus. Br J Surg. 1988;75(2):113–5.

62. Bremner CG, Lynch VP, Ellis Jr FH. Barrett's esophagus: congenital or acquired? An experimental study of esophageal mucosal regeneration in the dog. Surgery. 1970;68(1):209–16.
63. Wong J, Finckh ES. Heterotopia and ectopia of gastric epithelium produced by mucosal wounding in the rat. Gastroenterology. 1971;60(2):279–87.
64. Spechler SJ, Schimmel EM, Dalton JW, Doos W, Trier JS. Barrett's epithelium complicating lye ingestion with sparing of the distal esophagus. Gastroenterology. 1981;81(3):580–3.
65. Sartori S, Nielsen I, Indelli M, Trevisani L, Pazzi P, Grandi E. Barrett esophagus after chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF): an iatrogenic injury? Ann Intern Med. 1991;114(3):210–1.
66. Collen MJ, Lewis JH, Benjamin SB. Gastric acid hypersecretion in refractory gastroesophageal reflux disease. Gastroenterology. 1990;98(3):654–61.
67. Stein HJ, Eypasch EP, DeMeester TR, Smyrk TC, Attwood SE. Circadian esophageal motor function in patients with gastroesophageal reflux disease. Surgery. 1990;108(4):769–77. discussion 77-8.
68. Lavery DL, Nicholson AM, Poulsom R, Jeffery R, Hussain A, Gay LJ, et al. The stem cell organisation, and the proliferative and gene expression profile of Barrett's epithelium, replicates pyloric-type gastric glands. Gut. 2014;63(12):1854–63.
69. Seery JP. Stem cells of the oesophageal epithelium. J Cell Sci. 2002;115:1783–9.
70. Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science. 2012;337(6095):730–5.
71. Merlos-Suarez A, Barriga FM, Jung P, Iglesias M, Cespedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell. 2011;8(5):511–24.
72. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell. 2010;6(1):25–36.
73. Moyes LH, McEwan H, Radulescu S, Pawlikowski J, Lamm CG, Nixon C, et al. Activation of Wnt signalling promotes development of dysplasia in Barrett's oesophagus. J Pathol. 2012;228(1):99–112.
74. Smith RR, Hamilton SR, Boitnott JK, Rogers EL. The spectrum of carcinoma arising in Barrett's esophagus. A clinicopathologic study of 26 patients. Am J Surg Pathol. 1984;8(8):563–73.
75. Skinner DB, Walther BC, Riddell RH, Schmidt H, Iascone C, DeMeester TR. Barrett's esophagus. Comparison of benign and malignant cases. Ann Surg. 1983;198(4):554–65.
76. Bhat S, Coleman HG, Yousef F, Johnston BT, McManus DT, Gavin AT, et al. Risk of malignant progression in Barrett's esophagus patients: results from a large population-based study. J Natl Cancer Inst. 2011;103(13):1049–57.
77. McDonald SA, Greaves LC, Gutierrez-Gonzalez L, Rodriguez-Justo M, Deheragoda M, Leedham SJ, et al. Mechanisms of field cancerization in the human stomach: the expansion and spread of mutated gastric stem cells. Gastroenterology. 2008;134(2):500–10.
78. Snippert HJ, Schepers AG, van Es JH, Simons BD, Clevers H. Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. EMBO Rep. 2014;15(1):62–9.
79. Slack JM. Metaplasia and transdifferentiation: from pure biology to the clinic. Nat Rev Mol Cell Biol. 2007;8(5):369–78.
80. Wang X, Ouyang H, Yamamoto Y, Kumar P, Wei T, Dagher R, Vincent M, Lu X, Bellizzi A, Ho KY, Crum C, Xian W, McKeon F. Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell. 2011;145:1023–35.
81. Lord RVN, Wickramasinghe K, Johansson JJ, et al. Cardiac mucosa in the remnant esophagus after esophagectomy is an acquired epithelium with Barrett's-like features. Surgery. 2004;136:633–40.

82. O'Riordan JM, Tucker ON, Byrne PJ, et al. Factors influencing the development of Barrett's epithelium in the esophageal remnant postesophagectomy. Am J Gastroenterol. 2004;99:205–11.
83. Chaves P, Pereira AD, Cruz C, et al. Recurrent columnar-lined esophageal segments-study of the phenotypic characteristics using intestinal markers. Dis Esophagus. 2002;15:282–6.
84. Dresner SM, Griffin SM, Wayman J, et al. Human model of duodenogastro-oesophageal reflux in the development of Barrett's metaplasia. Br J Surg. 2003;90:1120–8.
85. Lindahl H, Rintala R, Sariola H, et al. Cervical Barrett's esophagus: a common complication of gastric tube reconstruction. J Pediatr Surg. 1990;25:446–8.
86. Tobey NA, Argote CM, Awayda MS, et al. Effect of luminal acidity on the apical cation channel in rabbit esophageal epithelium. AMJ Physiol Gastrointest Liver Physiol. 2007;292:G796–805.
87. Dixon J, Strugala V, Griffin SM, et al. Esophageal mucin: an adherent mucus gel barrier is absent in the normal esophagus but present in columnar-lined Barrett's esophagus. Am J Gastroenterol. 2001;96:2575–83.
88. Jovov B, Van Itallie CM, Shaheen NJ, et al. Claudin-18: a dominant tight junction protein in Barrett's esophagus and likely contributor to its acid resistance. Am J Physiol Gastrointest Liver Physiol. 2007;293:G1106–13.
89. Coad RA, Woodman AC, Warner PJ, Barr H, Wright NA, Shepherd NA. On the histogenesis of Barrett's oesophagus and its associated squamous islands: a three-dimensional study of their morphological relationship with native oesophageal gland ducts. J Pathol. 2005;206(4):388–94.
90. Barbera M, Fitzgerald RC. Cellular origin of Barrett's metaplasia and oesophageal stem cells. Biochem Soc Trans. 2010;38(2):370–3.
91. Sarosi G, Brown G, Jaiswal K, Feagins LA, Lee E, Crook TW, et al. Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus. Dis Esophagus. 2008;21(1):43–50.
92. Okamoto R, Yajima T, Yamazaki M, et al. Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med. 2002;8:1011–7.
93. Aikou S, Aida J, Takubo K, Yamagata Y, Seto Y, Kaminishi M, Nomura S. Columnar metaplasia in a surgical mouse model of gastro-esophageal reflux disease is not derived from bone-marrow cell. Cancer Sci. 2013;104:1154–61.
94. Barbera M, di Pietro M, Walker E, Brierley C, MacRae S, Simons BD, Jones PH, Stingl J, Fitzgerald RC. The human squamous oesophagus has widespread capacity for clonal expansion from cells at diverse stages of differentiation. Gut. 2015;64:11–9.
95. Groisman GM, Amar M, Meir A. Expression of the intestinal marker Cdx2 in the columnar-lined esophagus with and without intestinal (Barrett's) metaplasia. Mod Pathol. 2004;17(10):1282–8.
96. Phillips RW, Frierson Jr HF, Moskaluk CA. Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. Am J Surg Pathol. 2003;27(11):1442–7.
97. Going JJ, Fletcher-Monaghan AJ, Neilson L, et al. Zoning of mucosal phenotype, dysplasia, and telomerase activity measured by telomerase repeat assay protocol in Barrett's esophagus. Neoplasia. 2004;6:85–92.
98. Chandrasoma P, Lokuhetty DM, Demeester TR, et al. Definition of histopathologic changes in gastroesophageal reflux disease. Am J Surg Pathol. 2000;24:344–51.
99. Guo R-J, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. Cancer Biol Ther. 2004;3:593–601.
100. Souza RF, Krishnan K, Spechler SJ. Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. Am J Physiol Gastrointest Liver Physiol. 2008;295:G211–8.
101. Theodorou D, Ayazi S, Demeester SR, et al. Intraluminal pH and goblet cell density in Barrett's esophagus. J Gastrointest Surg. 2012;16:469–74.
102. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Peterson OW. The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation. 2002;70(9–10):537–46.

103. Sharma P, Dent J, Armstrong D, Bergman JJGHM, Gossner L, Hoshihara Y, et al. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. Gastroenterology. 2006;131(5):1392–9.
104. Aida J, Vieth M, Ell C, May A, Pech O, Hoshihara Y, et al. Palisade vessels as a new histologic marker of esophageal origin in ER specimens from columnar-lined esophagus. Am J Surg Pathol. 2011;35(8):1140–5.
105. Hackelsberger A, Günther T, Schultze V, Manes G, Dominguez-Muñoz J-E, Roessner A, et al. Intestinal metaplasia at the gastro-oesophageal junction:Helicobacter pylori gastritis or gastro-oesophageal reflux disease? Gut. 1998;43(1):17–21.
106. Goldblum JR, Richter JE, Vaezi M, Falk GW, Rice TW, Peek RM. Helicobacter pylori infection, not gastroesophageal reflux, is the major cause of inflammation and intestinal metaplasia of gastric cardiac mucosa. Am J Gastroenterol. 2002;97(2):302–11.
107. Levine DS. Management of dysplasia in the columnar-lined esophagus. Gastroenterol Clin North Am. 1997;26(3):613–34.
108. Curvers WL, Singh R, Song LM, Wolfsen HC, Ragunath K, Wang K, et al. Endoscopic trimodal imaging for detection of early neoplasia in Barrett's oesophagus: a multi-centre feasibility study using high-resolution endoscopy, autofluorescence imaging and narrow band imaging incorporated in one endoscopy system. Gut. 2008;57(2):167–72.
109. Pohl J, Pech O, May A, Manner H, Fissler-Eckhoff A, Ell C. Incidence of macroscopically occult neoplasias in Barrett's esophagus: are random biopsies dispensable in the era of advanced endoscopic imaging? Am J Gastroenterol. 2010;105(11):2350–6.
110. Alverez Herrero L, Curvers WL, Bansal A, Wani S, Kara M, Schenk E, et al. Zooming in on Barrett oesophagus using narrow-band imaging: an international observer agreement study. Eur J Gastroenterol Hepatol. 2009;21(9):1068–75.
111. Takubo K, Nixon JM, Jass JR. Ducts of esophageal glands proper and paneth cells in Barrett's esophagus: frequency in biopsy specimens. Pathology. 1995;27(4):315–7.
112. Gore S, Healey CJ, Sutton CJ, Eyre-Brook IA, Gear MWL, Shepherd NA, Wilkinson SP. Regression of columnar line (Barrett's oesophagus) with continuous omeprazole therapy. Aliment Pharmacol Ther. 1993;7:623–8.
113. Malesci A, Savarino V, Zentilin P, Belicchi M, Mela GS, Lapertosa G, Bocchio P, Ronchi G, Franceschi M. Partial regression of Barrett's esophagus by long term therapy with high-dose omeprazole. Gastrointest Endosc. 1996;44:700–5.
114. Shields HM, Rosenberg SJ, Zwas FR, Ransil BJ, Lembo AJ, Odze R. Prospective evaluation of multilayered epithelium in Barrett's esophagus. Am J Gastroenterol. 2001;96(12):3268–73.
115. Glickman JN, Chen YY, Wang HH, Antonioli DA, Odze RD. Phenotypic characteristics of a distinctive multilayered epithelium suggests that it is a precursor in the development of Barrett's esophagus. Am J Surg Pathol. 2001;25(5):569–78.
116. Srivastava A, Odze RD, Lauwers GY, Redston M, Antonioli DA, Glickman JN. Morphologic features are useful in distinguishing Barrett esophagus from carditis with intestinal metaplasia. Am J Surg Pathol. 2007;31(11):1733–41.
117. Shi L, Der R, Ma Y, Peters J, Demeester T, Chandrasoma P. Gland ducts and multilayered epithelium in mucosal biopsies from gastroesophageal-junction region are useful in characterizing esophageal location. Dis Esophagus. 2005;18(2):87–92.
118. Takubo K, Aida J, Sawabe M, Arai T, Kato H, Pech O, et al. The normal anatomy around the oesophagogastric junction: a histopathologic view and its correlation with endoscopy. Best Pract Res Clin Gastroenterol. 2008;22(4):569–83.
119. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association technical review on the management of Barrett's esophagus. Gastroenterology. 2011;140(3):e18–52. quiz e13.
120. Chandrasoma PT, Der R, Dalton P, et al. Distribution and significance of epithelial types in columnar-lined esophagus. Am J Surg Pathol. 2001;25:1188–93.
121. Liu W, Hahn H, Odze RD, et al. Metaplastic esophageal columnar epithelium without goblet cells shows DNA content abnormalities similar to goblet cell containing epithelium. Am J Gastroenterol. 2009;104:816–24.

122. Rubio CA, Riddell R. Muculo-fibrous anomaly in Barrett's mucosa with dysplasia. Am J Surg Pathol. 1988;12:885–9.
123. Takubo K, Sasajima K, Yamashita K, et al. Double muscularis mucosae in Barrett's esophagus. Hum Pathol. 1991;22:1158–61.
124. Abraham SC, Krasinskas AM, Correa AM, et al. Duplication of the muscularis mucosae in Barrett esophagus: an underrecognized feature and its implication for staging of adenocarcinoma. Am J Surg Pathol. 2007;31:1719–25.
125. Tang P, MicKinley MJ, Sporrer M, et al. Inlet patch: prevalence, histologic type, and association with esophagitis, Barrett esophagus, and antritis. Arch Pathol Lab Med. 2004;128:444–7.
126. Gutierrez O, Akamatsu T, Cardona H, Graham DY, El-Zimaity HM. Helicobacter pylori and heterotopic gastric mucosa in the upper esophagus (the inlet patch). Am J Gastroenterol. 2003;98(6):1266–70.
127. Ormsby AH, Goldblum JR, Rice TW, Richter JE, Falk GW, Vaezi MF, Gramlich TL. Cytokeratin subsets can reliably distinguish Barrett's esophagus from intestinal metaplasia of the stomach. Hum Pathol. 1999;30:288–94.
128. Glickman JN, Wang H, Das KM, Goyal RK, Spechler SJ, Antonioli D, Odze RD. Phenotype of Barrett's esophagus and intestinal metaplasia of the distal esophagus and gastroesophageal junction: an immunohistochemical study of ytokeratins 7 and 20, Das-I and 45 MI. Am J Surg Pathol. 2001;25:87–94.
129. Ormsby AH, Vaezi MF, Richter JE, Goldblum JR, Rice TW, Falk GW, Gramlich TL. Cytokeratin immunoreactivity patterns in the diagnosis of short-segment Barrett's esophagus. Gastroenterology. 2000;119:683–90.
130. Lorinc E, Jakobsson B, Landberg G, Veress B. Ki67 and p53 immunohistochemistry reduces interobserver variation in assessment of Barrett's oesophagus. Histopathology. 2005;46:642–8.

Chapter 8
What Makes an Expert Barrett's Histopathologist?

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Introduction

Progression to dysplasia and cancer in Barrett's esophagus (BO) is driven by recurrent genetic mutation and waves of clonal expansion. However, great clinical heterogeneity is observed in the tempo and mode of malignant progression: whereas some patients show small invasive lesions without antecedent dysplasia in the surrounding mucosa, other patients show extensive dysplastic change apparently without progression to invasive disease. The reasons underlying this clinical heterogeneity remain unclear. There is also tremendous phenotypic (morphologic) heterogeneity in BO progression. In part this is due to the inflammatory nature of the disease, characterized by continuous cycles of inflammation and epithelial regeneration, which severely complicates histopathological assessment of biopsy material. Together these clinical and histopathologic issues frustrate efforts aimed at risk stratification in BO. Currently, most guidelines and consensus meetings aim to decrease surveillance frequency in patients

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with "benign" BO compared to those with a higher risk of malignant progression [1–3]. In this model of escalation and deescalation, high quality histopathology workup is essential. Endoscopic resection, often in combination with ablative treatment, is now widely undertaken for early stage disease and has proven to be a safe and effective treatment.

Successful treatment and follow-up depends on close collaboration between endoscopists and pathologists. For the histopathologist, familiarity with clinical advances deserves constant attention. The endoscopist, on the other hand, should be familiar with areas of diagnostic difficulty in histopathology and understand limitations inherent to histopathologic workup. Here we discuss a diagnostic algorithm and provide examples of frequently encountered diagnostic pitfalls in biopsy and endoscopic resection material. In this chapter, BO is equivalent to columnar lined esophagus (CLO) as diagnosed by the endoscopist (discussed in the accompanying manuscript by Neil Shepherd and colleagues Chap. 7).

General Approach

The classification of dysplasia in BO has traditionally been based on the approach to dysplasia classification in inflammatory bowel disease (IBD) [4]. This latter classification has evolved over the years and, in an attempt to minimize diagnostic differences between Western and Eastern histopathologists, the Vienna classification was created, which is shown in Table 8.1 [5]. Core features used to assess BO biopsies have been extensively described [5–7] and include surface maturation, glandular architecture, and cytonuclear changes (Table 8.2) [7, 8]. These features are surveyed at low magnification and confirmed at higher magnification. Since few features in differentiating "absence of dysplasia" from bona fide dysplasia can said to be unambiguous, in general a combination of features is needed to arrive at a final diagnosis. Several key features are discussed next.

Table 8.1 Vienna classification of gastrointestinal epithelial neoplasms

Category	Description
1	Negative for dysplasia/neoplasia
2	Indefinite for dysplasia/neoplasia
3	Noninvasive[a] low-grade neoplasia (low-grade adenoma/dysplasia)
4	Noninvasive high-grade neoplasia 4.1 High-grade adenoma/dysplasia 4.2 Noninvasive carcinoma (carcinoma in situ) 4.3 Suspicion of invasive carcinoma
5	Invasive neoplasia 5.1 Intramucosal carcinoma 5.2 Submucosal[b] carcinoma or beyond

[a]Noninvasive indicating absence of invasion
[b]Submucosal indicating invasion into lamina propria or beyond

Table 8.2 Diagnostic algorithm

1	*Clonality*	Are transitions within the biopsy abrupt or gradual?
2	*Surface maturation*	Is surface maturation present within the biopsy? If not, why?
3	*Architecture*	Is the architectural complexity within the normal/regenerative range or is it due to neoplastic proliferation?
4	*Cytonuclear features*	Are nuclear changes in keeping with metaplasia, regeneration, or inflammation? Or are they due to dysplasia?
5	*Inflammation*	Are the alterations driven by inflammation or secondary to a clonal process?

Clonal Transitions in BO Dysplasia

A key feature in the approach to differentiating neoplastic from reactive change in BO is whether the epithelium shows a "clonal transition." Neoplastic proliferation is driven by oncogenic mutations (such as *TP53* point mutations), which have accumulated in the genomes of stem cells in the epithelium. If these genetic changes provide a fitness advantage (i.e., faster proliferation, resistance to apoptosis, etc.) then the descendants of cells that incurred these mutations will clonally expand within the neighboring epithelium. Presumably, many genetic mutations will subtly alter the fitness of stem cells and their direct descendants, but the majority of these mutations will not provoke a phenotypic change and will therefore not be apparent in routine microscopy. Some mutations (such as biallelic *TP53* mutations), however, will result in a phenotypic change morphologically apparent in routine microscopy. These morphological changes are really a surrogate marker of genetic clonal expansions in BO. Because genetic mutations are indelible, permanent agents of change, the resultant phenotypic change will be present throughout the clonal expansion as an "all or none" phenomenon. For this reason, neoplastic change in BO will be morphologically sharply demarcated from the nonneoplastic mucosa. This "clonal" step change can often be appreciated in dysplastic BO mucosa at low magnification and at higher magnification it is often possible to point to the exact boundary between dysplastic and nondysplastic epithelium at single cell resolution. This contrasts with reactive changes due to inflammation, where the proliferation and the accompanying microscopic alterations are a response to inflammatory stimuli and are therefore not governed by genetic mutations. These reactive changes are morphologically less uniform, not as sharply demarcated, and gradually fade in prominence moving away from the epithelial insult.

Surface Maturation

Normal epithelial proliferation and regeneration in nondysplastic BO takes place in the deeper parts of the glands. These deeper parts of the gland are usually packed somewhat closer together and show a higher nuclear/cytoplasmic (N/C) ratio and hyperchromatic ("darker") nuclei on H&E. As the epithelium differentiates and

migrates toward the luminal surface, the nuclei become smaller, less hyperchromatic and the cytoplasmic volume increases, resulting in an altogether lighter tone to the mucosa. This phenomenon of zonation can disappear (known as "loss of maturation") when there are increased numbers of cells and nuclei at the mucosal surface. This increase can be caused by either reactive stimuli (inflammation and regeneration) or neoplastic proliferation, or a combination of both. If surface maturation is not easily identified when assessing a biopsy on overview, the underlying cause should always be clarified.

Architecture

The architectural features of the mucosa in a biopsy are determined by the interrelationships between the number and the shape of the glands and the surrounding lamina propria. Nondysplastic glands tend to be uniformly round tubules surrounded by lamina propria. In the context of inflammation and regeneration, more variation in glandular shape and size is expected. Abnormal architectural features include changes in the shape of glands (such as irregular contours or cystic dilatation) as well as changes in the number of glands compared to the amount of surrounding stroma. In dysplasia, abnormal architectural arrangement is invariably accompanied by cytonuclear abnormalities. Some architectural abnormalities are relatively specific for neoplasia, such as true "back-to-back" configurations or cribriform growth patterns. These changes are often best appreciated when compared to the architectural arrangement of neighboring nondysplastic gastric or intestinal metaplastic glands.

Cytonuclear Atypia

Some (reactive) cytonuclear atypia is inherent to the inflammatory nature of BO. Size and shape of nuclei are assessed in relation to their location in the gland and compared to the surrounding glands or surface epithelium. In general, larger nuclei are more readily accepted when they are located deeper in the glands, rather than at the mucosal surface. At the surface many nonneoplastic conditions can give the impression of (pseudo-)stratification (more about this later), including inflammatory, regenerative, and dysplastic changes. Inflammation can cause severe architectural and often worrisome cytonuclear abnormalities, mimicking dysplasia and the differential diagnosis in these cases encompasses both low- and high-grade dysplasia (LGD and HGD, respectively).

In summary, architectural and cytonuclear alterations in BO can occur both in the context of reactive changes associated with inflammation and in the context of bona fide dysplasia. The four features described earlier are integrated and weighed against the inflammatory component (if present) to attain a definitive diagnosis. In the following paragraphs, we describe the distinction between

dysplasia and regenerative change, before discussing LGD and HGD in biopsies. The category of "indefinite for dysplasia" is discussed separately.

Negative for Dysplasia

This ranges from normal, unremarkable metaplastic BO to epithelium with extreme regenerative features due to heavy active inflammation. At any point along this spectrum, one may consider dysplasia or sometimes even malignant transformation (Fig. 8.1). With this in mind, it is important to consider the observed epithelial changes in their proper context and be mindful of possible pitfalls. In general, surface maturation and architecture remain intact in noninflamed, nondysplastic BO. Surface epithelium shows a regularly undulating or slightly villiform architectural arrangement and the lining cells range from a columnar foveolar type to intestinal metaplastic type, or a combination of both. The glands in the deeper part of the specimen contain more uniform and rounded glands of either cardiac, fundic, or intestinal-type mucosa and are regularly dispersed in ample intervening lamina propria. Junctional or cardiac-type epithelium contains bland looking mucus-secreting columnar cells resembling cardiac or pyloric type glands. In fundic type epithelium, both parietal and chief cells are observed, while specialized intestinal metaplasia demonstrates goblet cells. Sometimes pancreatic metaplasia can be observed. The most common forms of metaplasia encountered in specimens are intestinal and junctional-type epithelium, but all epithelial types and architectural patterns listed earlier can be observed together in varying proportions. Glands displaying intestinal metaplasia in nondysplastic mucosa usually stand out more than gastric-type mucosa. The deeper area of these glands showing intestinal metaplasia contains the

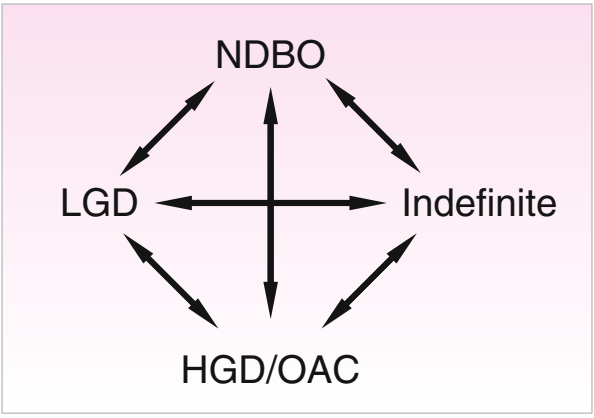

Fig. 8.1 Nonlinear differential diagnostic possibilities in Barrett's esophagus. *NDBO* nondysplastic Barrett's esophagus, *IND* indefinite for dysplasia, *LGD* low-grade dysplasia, *HGD* high-grade dysplasia, *OAC* esophageal adenocarcinoma

proliferative compartment and multiple mitoses are to be expected. Mitotic figures at the luminal surface of the gland, however, are usually not observed and these should be treated with caution. The most frequently encountered diagnostic difficulties when discerning nondysplastic from dysplastic tissue include loss of surface maturation, changes at the squamo-columnar junction, pseudo-stratification of nuclei at the surface epithelium, and epithelial changes in the context of inflammation. Often a combination of these factors is observed.

Due to the underlying repetitive inflammation and regeneration in BO, reactive epithelial changes are most pronounced at the squamo-columnar junction (Fig. 8.2a) and should not be overinterpreted as dysplasia. Reactive surface epi-

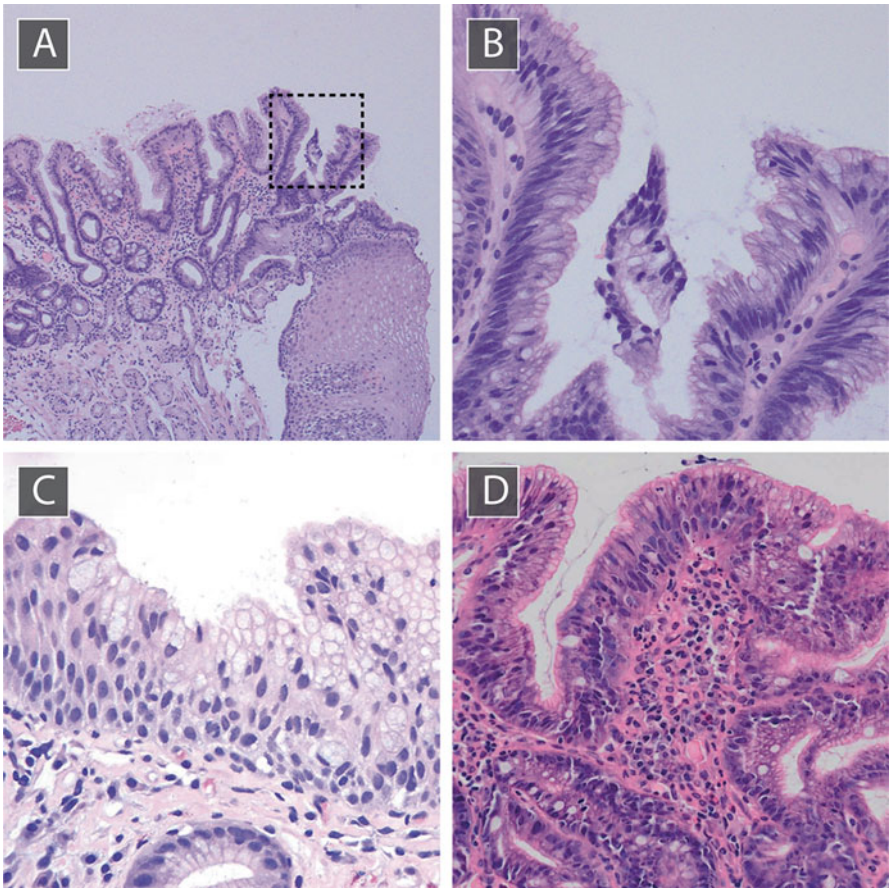

Fig. 8.2 (**a**) The heterogeneous aspect at the squamo-columnar junction on overview can give the impression of dysplasia-associated stratification of nuclei at the surface and loss of maturation. (**b**) Detail of figure (**a**) (see *dashed box*) showing characteristic reactive changes consistent with pseudo-stratification. (**c**) Multilayered epithelium in a specimen of nondysplastic Barrett's, displaying the transition from squamous epithelium (*left*) to pseudo-stratified columnar cells (*right*). (**d**) Heavy active inflammation resulting in extensive reactive changes in a biopsy of nondysplastic Barrett's esophagus

thelium characteristically consists of two rows of nuclei embedded in basophilic cytoplasm which is highlighted with an apical rim of clear cytoplasm (Fig. 8.2b). Cellular membranes of these pseudo-stratified cells are ill defined, which gives this epithelium a confluent or syncytial appearance. Several types of epithelium with multiple layers are recognized in BO and may mimic cellular crowding as observed in bona fide dysplasia. Multilayered epithelium consists of multiple layers of squamous cells covered with columnar, mucinous epithelium (Fig. 8.2c). The number of squamous cell layers varies but may be limited to a single squamous cell layer covered by columnar, mucinous epithelial cells. Pseudo-stratification, usually located at the tips of villous projections, is caused by the chaotic coalescence of migrating cells from multiple directions onto the villous tip and is not indicative of neoplastic change. In pseudo-stratification all nuclei are apposed in immediate proximity of the epithelial basement membrane. Due to sectioning artifacts however, nuclei may appear to pile up within the epithelium. By contrast, in "true" (i.e., dysplastic) nuclear stratification, only the bottom layer of nuclei remains directly apposed to the epithelial basement membrane. In daily practice the distinction between nuclear pseudo-stratification and "true" nuclear stratification is often challenging or sometimes impossible to make with certainty. Besides chronic inflammation, BO often demonstrates active epithelial inflammation, which may provoke superficial mucin depletion, presumably as a protective response. This may in turn mimic dysplasia-associated disturbed surface maturation. Moreover, active inflammation can induce complex architectural configurations, such as an uneven distribution of glands, irregular glandular contours, or cystic change and an abnormally protrusive villiform surface (Fig. 8.2d). Intraluminal necrotic debris in dilated cystic glands is not a feature of benign inflammation and should always be treated with caution. High power inspection examining the cytologic ("single cell") changes can reveal hyperchromasia and conspicuous nucleoli and increase in mitotic activity is not unusual either. However, even though inflammation-induced cytonuclear atypia can be severe, nuclear contours remain smooth and the N/C ratio remains within normal limits. Active inflammation and regeneration can cause conspicuous and even worrisome architectural and cytological changes but these changes tend to gradually wax and wane in severity throughout the specimen and diagnostic criteria should be strictly applied in its presence before considering dysplasia.

Dysplasia

The biopsy is examined first at low magnification for features that immediately stand out from the general pattern. Due to loss of goblet cell differentiation or mucin depletion at the surface together with an increase in the number of nuclei, the deeper and superficial parts of the mucosa may take on a similar appearance (Fig. 8.3a). This loss of surface maturation can be caused by either inflammatory or neoplastic changes, as discussed earlier. However, the presence of active inflammation does

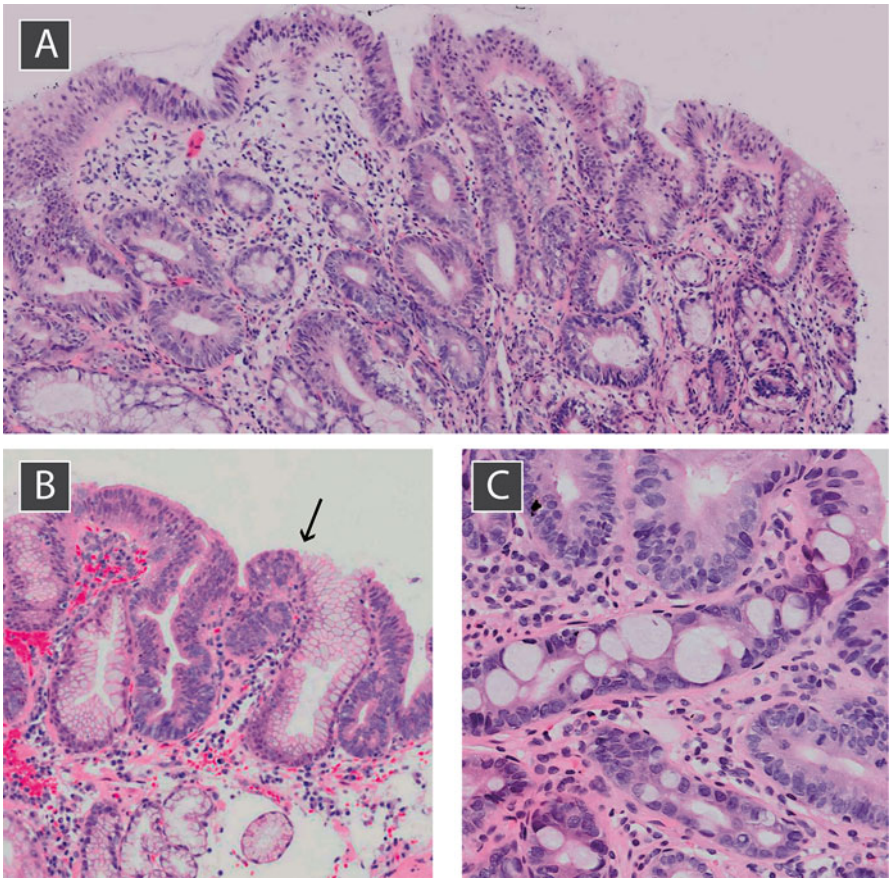

Fig. 8.3 (**a**) Overview of low-grade dysplasia with an overall intact architecture, but loss of surface maturation due to an increase in elongated nuclei in the dysplastic glands, which extends to the superficial part of the dysplastic gland. (**b**) Clonality in low-grade dysplasia, represented by a sharp, "clonal" transition in nuclear features from normal columnar cells to low-grade dysplasia (indicated by *arrow*). (**c**) Detail of a gland showing characteristic dystrophic goblet cells

not rule out the possibility of dysplasia and both can occur together. Abrupt transitions in architectural or nuclear features (i.e., either nuclear size or nuclear stratification) amount to "clonal changes" as discussed earlier and strongly favor true neoplastic change over reactive/regenerative change (Fig. 8.3b). Dysplastic changes may be either focal or extensive and in these latter cases come to occupy the entire mucosal surface. Generally speaking, the architectural and cytonuclear changes in LGD are less pronounced compared to those in HGD. However, the grade of dysplasia in BO is a continuous spectrum of architectural and cytonuclear abnormalities, rather than sharply delineated categories.

Low-Grade Dysplasia

The main feature of LGD is loss of surface maturation due to nuclear stratification at the glandular surface and retained overall glandular architecture (Fig. 8.3a, b). The number of goblet cells is frequently diminished and some glandular crowding is observed, although strands of mesenchymal tissue are still present between dysplastic glands. These stratified nuclei are mildly enlarged, hyperchromatic and elongated, and usually of uniform size and shape without nuclear membrane irregularities. In general, cellular apico-basal polarity is retained and loss of polarity of stratified nuclei is therefore more in keeping with HGD. However, dystrophic goblet cells are frequently observed in LGD. These are goblet cells that have lost cellular polarity and display an "upside down" or otherwise misoriented mucin bubble (Fig. 8.3c). Other architectural disturbances associated with LGD are more extensive glandular crowding or back-to-back localization and some cystic change, as long as accompanying cytonuclear features remain within limits in keeping with LGD.

High-Grade Dysplasia

In HGD architectural abnormalities are more pronounced and mitotic figures may extend to the surface epithelium. Complex architectural changes are present and include glandular branching or budding and cribriform growth ("gland-in-gland formation") (Fig. 8.4a). Cystic change in deeper parts of the mucosa with intraluminal necrotic debris is a distinct feature of HGD (Fig. 8.4b). Surface maturation is generally absent, but stratification of elongated nuclei at the surface (as is often observed in LGD) is not typical of HGD. Instead, a single layer of cells with marked cytonuclear atypia is more often observed (Fig. 8.4a). Deeper in the mucosa, the N/C ratio is disturbed and the nuclear size is increased with nucleoli that are often, but not necessarily, prominent [6]. Nuclear shape is irregular and loss of nuclear polarity is frequently observed (Fig. 8.4c). One special type of HGD is the nonadenomatous or small cell type of dysplasia [6, 9]. This type demonstrates a monolayer of small cuboidal to columnar cells with remarkably smooth nuclear contours. These cells contain mucinous cytoplasm with monotonous small rounded and hyperchromatic nuclei (Fig. 8.4d). It is important to recognize this high-grade lesion in spite of its somewhat bland appearance. Some researchers have tried to define a minimum number of involved glands showing high-grade characteristics in order to justify a diagnosis of HGD [10]; others accept focal high-grade features as sufficient to warrant a HGD diagnosis. To date, no specific biomarker or panel of biomarkers is available for exact discrimination between LGD and HGD. Finally, it is not uncommon to find high-grade epithelial alterations without a clear low-grade precursor. We therefore stress that all of the features listed earlier are investigated regardless of clinical query.

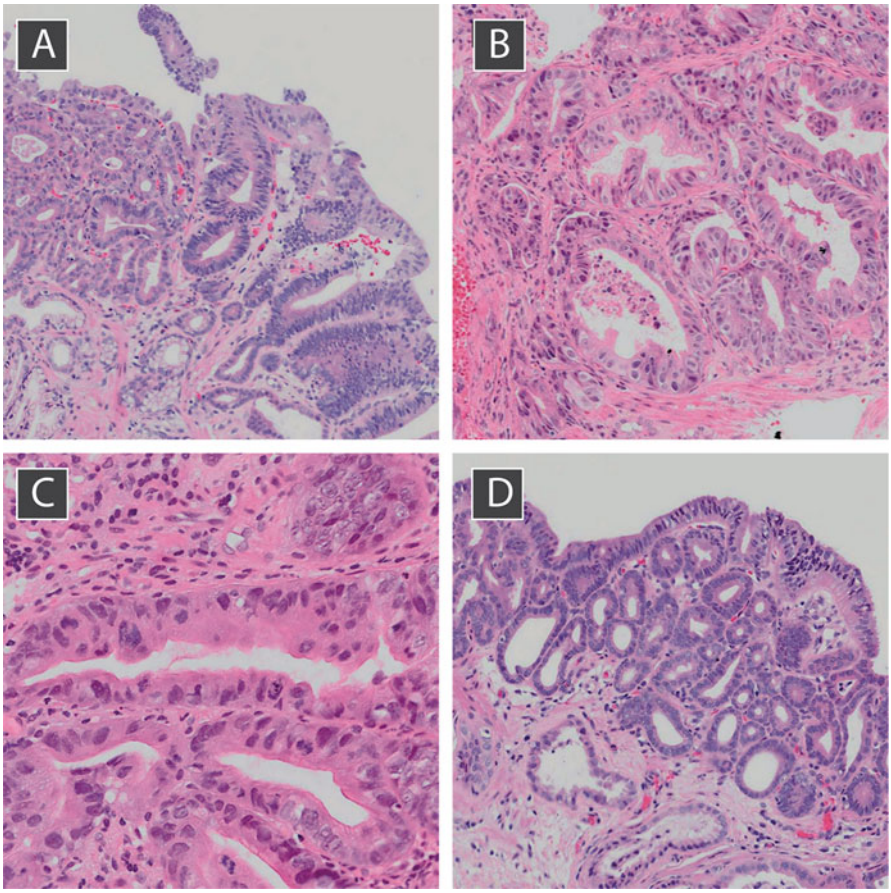

Fig. 8.4 (**a**) Overview of a biopsy with low-grade dysplasia (to the *right*) characterized by elongated but nonetheless regular nuclei, to be contrasted with high-grade dysplasia (on the *left*) demonstrating an altogether more complex growth pattern, back-to-back arraying of glands, loss of polarity, and polymorphic nuclei. (**b**) High-grade dysplasia showing a dilated gland containing intraluminal necrotic debris. (**c**) Detail of a dysplastic gland (high-grade features) with loss of polarity, complex architectural changes, and polymorphic nuclei. (**d**) Overview of small cell type high-grade dysplasia. The dysplastic glands are lined by a single layer of small, cuboidal-to-columnar cells with smooth nuclear contours

Indefinite for Dysplasia

In the Vienna classification system, the indefinite for dysplasia (IND) category is wedged between the "negative for dysplasia" and LGD category. We (argumentatively) think this has wrongly fostered the idea that IND is a label that can be applied to those cases that show some atypia, which is altogether insufficient for a diagnosis of LGD. It is important to underscore that the IND category does NOT represent a

grey-zone continuum between nondysplastic BO and LGD (Fig. 8.1). Lesions such as those described earlier which reveal some atypia but after applying the criteria described insufficient for a diagnosis of bona fide dysplasia should be placed in the "negative for dysplasia" category. When properly used, IND is a useful category reserved for those cases when a reliable distinction between nonneoplastic and neoplastic is not possible.

The differential diagnosis for IND cases extends from nondysplastic BO to LGD (most usual scenario), but also from reactive changes to HGD or even frank adenocarcinoma. Unfamiliarity with epithelial changes due to active inflammation and regenerative features may cause diagnostic uncertainty and be overdiagnosed as IND or neoplasia. Technical problems such as poor orientation, tangential cutting, marked cautery artifacts, or denuded surface epithelium can hamper proper diagnostics, mostly due to the inability to appreciate surface maturation. This should be clearly (unequivocally) recorded in the written report, when these technical issues compound the diagnostic dilemma.

If, after exhausting the complete diagnostic arsenal (including a second opinion by a colleague pathologist, additional immunohistochemistry and possible mutational screening), one remains in limbo over a firm diagnosis then we are confident that the IND category is justified. IND should not become a waste bucket diagnosis for those lesions, which only at first sight defy classification.

Controversy Regarding the "Indefinite for Dysplasia" Category

The broad spectrum of differential diagnostic considerations in the "IND" category can be confusing to the clinician. Even among histopathologists, the use of this diagnostic category is controversial. Some avoid its use altogether and will suggest acid suppression aimed at decreasing inflammatory changes before issuing a rebiopsy. Others consider it a very important and useful diagnostic category when a definitive diagnosis cannot be established, but neoplastic changes cannot be ruled out either. The significance of this diagnostic category mainly lies in its use as a warning signal to the endoscopist that this patient should not be deprived of endoscopic surveillance.

Few studies have tried to specifically address the prognostic implication of an IND diagnosis in BO follow-up [11–13]. In general, these studies affirm an increased risk for developing unequivocal neoplasia in patients with IND. However, explicit criteria for the diagnosis of IND are often not stated, complicating comparison between cohorts. Even when criteria are stated these often refer to those used by others for "crypt dysplasia" and "dysplasia" with preserved surface maturation [14]. To conclude, IND remains a challenging diagnostic entity and a difficult (and to a certain extent controversial) subject to study. Multiple diagnostic algorithms have been proposed to overcome these difficulties [10, 15], and besides a second opinion by a gastrointestinal (GI) pathologist, in our opinion, judicial use of *TP53* IHC can lend further credence to a dysplasia diagnosis in difficult cases, as described later.

Making the Most of P53 IHC

TP53 inactivation is by far the most common genetic alteration in BO dysplasia and OAC [16] Biallelic mutation of the *TP53* gene may result in a protein with aberrant biochemical and immunohistochemical staining properties and in theory this should provide an excellent diagnostic tool. However, studies on the added value of immunohistochemical staining for P53 have not shown unequivocal results. Indeed, current British guidelines advocate the use of P53 IHC stating that it would increase interobserver agreement, while American guidelines protest against the use of any additional molecular marker [2, 17, 18]. We feel that this may in part relate to the use of different protocols and, importantly, different antibody (cocktails) which recognize different epitopes. At this point immunohistochemistry with p53 may be the only adjunct marker of potential use in the clinical diagnostic setting. In our hands a cocktail of two monoclonal antibodies (BP53-11, Thermo scientific) is very reliable. We employ a protocol using antigen retrieval (in Tris–EDTA, pH 9.1) and a dedicated HRP amplification step. Details of our protocol are available upon request. This gives a highly sensitive and specific staining of *TP53*-mutant epithelium.

Genetic inactivation of *TP53* may result in either one of two aberrant patterns. The first of these is the so-called overexpression pattern. In this case an intense nuclear staining can be observed due to the accumulation of abnormal amounts of P53 protein (the protein product of the *TP53* locus) in the cell. The P53 protein in this case accumulates because the normal cellular feedback mechanisms remain intact. These pathways drive intense overexpression of the P53 protein in an effort to repair DNA damage. However, these signals are not relayed because the *TP53* node in the circuit has been rendered inactive, most commonly due to somatic point mutations in the protein's DNA-binding domain. Thus, the overexpression of the protein is a marker of the stress placed on the repair pathway. Other mechanisms such as an increased protein half-life of the mutant P53 protein may also contribute to its apparent overexpression. This first pattern is demonstrated in Fig. 8.5a. The second pattern is the so-called null mutation pattern. In this case there is a complete lack of P53 labeling in the dysplastic epithelium. This is either due to homozygous deletion of the *TP53* locus or due to mutations in the *TP53* transcript, which accelerate its degradation (nonsense mediated decay). Either way, there is no epitope for the antibody to recognize in the dysplastic epithelium, resulting in a complete lack of P53 labeling. In this case only the normal nuclear hematoxylin counterstain is seen. This second pattern can be seen in Fig. 8.5b. Importantly, note that normal epithelium, either normal squamous or non-dysplastic columnar epithelium invariably expresses a low background amount of P53 protein in the epithelial progenitor zone (i.e., the parabasal squamous cells or the cells in the glandular neck zone, respectively). This low background level of expression reflects ongoing normal DNA surveillance mechanisms, which are continuously monitoring the cellular response to DNA damage. This background level of labeling provides an internal P53 staining control and this can be seen in Fig. 8.5a, b (indicated by asterisks). It is estimated that between 10 and 20 % of dysplasia show the so-called null mutation pattern but not everybody is familiar with this (significant) absent

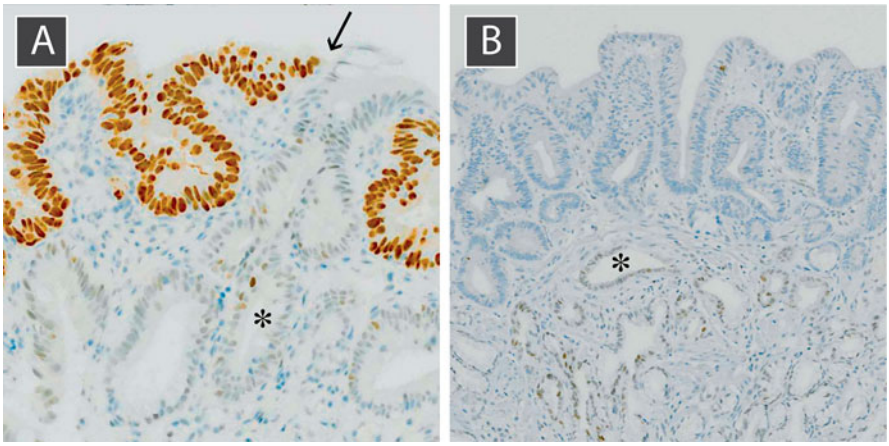

Fig. 8.5 (**a**) Overexpression of *TP53* in low-grade dysplasia marked by intense nuclear labeling pattern. By contrast, the normal background staining shows a variegated mix of weakly labeled brown and blue nuclei (*asterisk*). This reveals that "clonal transitions" in Barrett's progression can also be observed using immunohistochemistry (*arrow*). (**b**) Completely absent staining of p53 in low-grade dysplasia (so-called null mutation pattern), discernible as uniform strong blue staining (hematoxylin counterstain), which contrasts with the normal variegated background labeling pattern (*asterisk*)

staining pattern. P53 staining can be useful in IND cases if no consensus on the presence or absence of dysplasia is reached after consulting a colleague. When there is no consensus and no aberrant pattern is observed the diagnosis of IND is warranted. When there is no consensus and an aberrant pattern is observed a diagnosis of dysplasia can be considered. Note that a normal background-staining pattern in morphologically dysplastic epithelium does not exclude dysplasia and we stress that P53 IHC can support, but never supplant, proper diagnostic investigation of the H&E slide.

Interobserver Variability

Treatment and surveillance decisions in BO are mainly based on the presence and degree of dysplasia as determined by histological assessment of (surveillance) biopsies. No genetic biomarker has (yet) emerged that can replace histopathologic assessment [19, 20]. It has long been recognized, however, that the histologic diagnosis of dysplasia in BO is subject to considerable inter- and intraobserver variability [8].

The complete spectrum of dysplasia grades in BO is subject to significant interobserver variability, but this is particularly pronounced in the distinction between regenerative change and LGD. Studies have reported interobserver agreements for diagnosing LGD in BO that can be classified as poor to fair (k values ranging from 0.14 to 0.32) [8, 21–25]. Studies among GI pathologists with a special

interest in BO tend to demonstrate higher interobserver agreements for LGD, with *k* values ranging from 0.48 to 0.69 [15, 26–29], suggesting that experience and patient volume count in BO diagnostics. Likewise, several European studies have reported that risk stratification into low- and high-risk groups for neoplastic progression in BO is improved when histological review is performed by a dedicated GI pathologist [26–28, 30]. These studies included histological review, with agreement between panel pathologists in the upper range of reported interobserver agreements. Prospective studies from our institution have shown that the majority of community LGD diagnoses are downgraded after expert panel review to nondysplastic BO and these patients subsequently demonstrated a low risk of neoplastic progression (0–0.9 % per patient year of follow-up). On the other hand, when LGD was confirmed after expert consensus, the annual risk of progression to HGD and esophageal adenocarcinoma markedly increased (to 9.1–13.4 % per patient year of follow-up) [26, 27]. Comparable studies from the United States have shown markedly different results. In these studies considerably lower neoplastic progression rates were reported for confirmed low-grade dysplasia [21, 31]. In some cases the annual risk of progression of an LGD diagnosis was no different from the risk of progression to high-grade disease in nondysplastic BO [21]. The discrepancy between European and American studies is most likely related, at least in part, to the methodology of histology review, since a consensus diagnosis was not always included [31] and interobserver agreement between pathologists was low (*k* value of 0.14) [21]. In conclusion, histological review by a panel of dedicated BO pathologists can improve risk stratification. Therefore, current international guidelines recommend review of all LGD cases by a dedicated GI pathologist [1, 2, 32].

Endoscopic Treatment of High-Grade Lesions

The distinction between HGD and intramucosal carcinoma on biopsy material is similarly difficult and exact infiltration depth simply cannot be reliably determined on biopsy material even if a lesion is deemed to show infiltrative growth. Moreover, despite the Vienna classification, differences in diagnosing (early) invasive adenocarcinoma remain between pathologists in the Far East and the West. This is because histomorphologic features that define the earliest signs of invasion are not universally agreed upon. For example, the importance attributed to cytonuclear features in defining invasive biology varies geographically and this feature is used with greater emphasis by histopathologists in the Far East [33]. Diagnostic differences mostly apply to biopsy material and to a lesser extent to endoscopic resection specimens wherein features of well-differentiated mucosal carcinoma (such as intratubular lateral expansion and fusion of neighboring glands) can be more readily appreciated [34]. With widely available endoscopic resection modalities, this distinction on biopsy material between HGD and intramucosal cancer has however become less important from a management perspective and, after careful staging, both will be managed in a similar manner in experienced centers.

Handling the Endoscopic Resection Specimen

Endoscopic mucosal resection (EMR) specimens should be handled with utmost care. The specimen is loosely pinned onto cork, wax, or cardboard with the mucosa facing upward. This is performed in the endoscopy suite immediately following endoscopic retrieval of the specimen and before fixation. Pinning the specimen down freshly preserves tissue orientation and stops the lateral margins from folding over or curling up (see later). Avoid overstretching the specimen by pinning it down too tightly, since the specimen will shrink during fixation and this can cause tearing of the mucosa. This may also lead to incorrect depth of invasion measurements. The specimen is pinned down using fine tailor's needles. Preferably, the needles are not pinched through the (macroscopically) abnormal areas. Fixation in 4 % neutral buffered formalin takes place overnight or for a minimum period of 12 h. After fixation, the specimen is measured in three dimensions and the surface is scrutinized for visible lesions and the relation of the lesion (if macroscopically visible) to the closest lateral margins is appropriately assessed and recorded. EMR specimens are sometimes resected with a small tumor-free area surrounding the lesion and in some institutions a microscope with reverse lighting is used for detailed orientation and documentation of the specimen. The lateral and deep margins are inked and a macroscopic photomicrograph of the specimen is taken for orientation and mapping. In case the resection consists of multiple specimens, each specimen is measured and photographed separately. The specimen is "breadloafed" into 2–3 mm wide sections with the slicing direction allowing for optimal assessment of the most threatened lateral margin. Depending on the size of the specimen, the direction of slicing may be chosen parallel to its shortest axis, since long slices may twist during embedding and processing and this can result in noninterpretable histology slides. If the resection margin of interest is not optimally visible in this way, another slicing direction may be chosen. In larger resection specimens (for instance, endoscopic submucosal dissection (ESD) specimens), and depending on the distribution of visible lesions, divided perpendicular or *en face* sections of the resection margin may be chosen alternatively [35]. Clearly, the advantage of perpendicular sections is the circumferential visualization of the resection margins. This is particularly true in larger specimens. The drawback of this technique is that sections are often more difficult to interpret and in a focally positive resection margin, multiple step sections will be necessary before the definitive margin status can be determined. The slicing axis is recorded on the photomacrograph to avoid ambiguities. Obviously, the R0 status of the lateral margins cannot be adequately assessed if the resection was performed piecemeal. Consecutive slices of the specimen are placed into individual cassettes, with a maximum of three slices per cassette (but preferably less), the first and last slice (the polar ends) embedded separately. To allow for expertly orientated and optimally interpretable slides, slices are embedded and cut by a trained and dedicated technician.

The EMR Pathology Report

The pathology report includes the type(s) of surface epithelium present in the specimen as well as the status of the deepest tissue layer. When present, dysplasia is graded and lateral margin status is described. If an invasive lesion is present, the specimen is analyzed for four factors to assess the risk for recurrent disease and lymph node metastasis. These risk factors are as follows: differentiation grade, depth of invasion, resection margin status (R-status), and presence or absence of (lympho-) vascular invasion.

Differentiation Grade

Differentiation grade of (mucosal) adenocarcinoma is divided into well (G1), moderate (G2), or poor differentiation (G3), the latter correlating with a worse outcome since it increases the risk of (loco regional) lymph node metastasis (LNM). The WHO classification of tumors of the digestive system [36] stipulates that the most prominent differentiation pattern is taken into account for grading purposes, without acknowledging minor parts of poor differentiation or the nature of the invasive tumor front. Foci of poor differentiation, however, influence patient outcome even if the majority of the tumor is well differentiated [37]. In other organs, such as the prostate, the heterogeneity of tumor differentiation is explicitly taken into account by combining both the most prominent and the worst differentiation patterns for final grading (cf. Gleason score). Although poor differentiation grade is considered to be a risk factor for LNM in EMR specimens, the impact of minor foci of poor differentiation in a predominantly well or moderately differentiated lesion is currently unknown. Until this is resolved, we propose that both the most prominent and the worst differentiation grade are recorded in the (microscopy section of the) pathology report. This is preferably recorded as a percentage of total tumor volume. The most poorly differentiated part should be recognizable at low to medium magnification and this includes solid growth pattern, expansion as single cells, or signet cell differentiation.

Infiltration Depth

Esophageal cancers that have spread beyond the original muscularis mucosae are staged as T1b disease. Cancers that have not spread beyond the original muscularis mucosae are staged as T1a disease. Although this appears straightforward enough, multiple classification systems exist to subdivide T1a disease. This is mainly due to the presence of a duplicated muscularis mucosae and the weight that is given to penetration of this “neo-anatomic” boundary. One classification system is recommended by

the American Joint Committee on Cancer (AJCC), as described by Hölscher et al.[38]. Mucosal disease in this system is divided into three levels: either HGD (m1); or intramucosal carcinoma limited to the lamina propria (m2); or intramucosal carcinoma reaching within the muscularis mucosa (m3), regardless of whether this is the duplicated or original muscularis mucosae [38]. The second system by Westerterp et al. is similar, except for when invasion into the duplicated muscularis mucosae is present: invasion into the superficial layer is defined as m2, while invasion into the deeper (pre-existing) muscularis mucosae is defined as m3 [39]. The third system is propagated by Vieth and Stolte and also takes invasion into the duplicated muscularis mucosae into account [40]. However, the latter is a four-tiered system and classifies m1 as limited to the lamina propria, m2 as invasion into the superficial layer of the duplicated muscularis mucosae, m3 as invasion into the space between the original and the duplicated muscularis mucosae, and m4 as invasion into the deep (original) muscularis mucosae (Fig. 8.6). This system is based on the histoanatomical structures of BO. By providing a description of the exact histological layers this system also underscores the significance of the duplicated muscularis mucosa in BO biology [41].

Endoscopist and pathologist will need to mutually agree on the classification scheme to be used in order to avoid misunderstandings in interpreting the histopathology report. The system of the AJCC [38] and the system by Vieth and Stolte [40] are most frequently used, some authors even advocate to document findings according to both systems [42]. In order to be able to compare studies that make use of different classification systems, description of the exact depth of infiltration is crucial. This can be achieved by recording the exact histological layer.

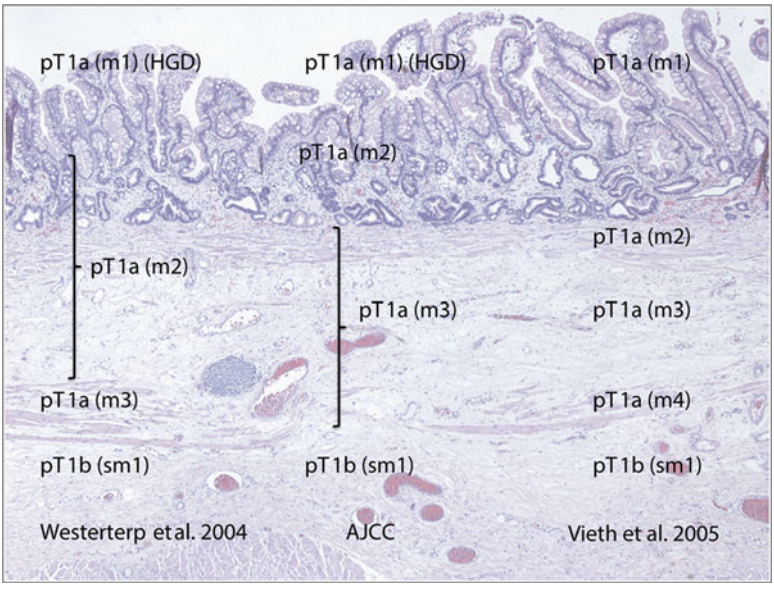

Fig. 8.6 Different classification systems used to stage mucosal adenocarcinoma in relation to the duplicated (superficial) and original (deep) muscularis mucosae. For details see text

The risk of LNM or disease recurrence increases significantly in patients with submucosal adenocarcinoma (T1b disease) [37]. Regardless of classification system, the invasion depth in the submucosa is subdivided as sm1-3. Importantly, this subdivision is based on the superficial, middle and deep 1/3 level of the submucosa as measured in esophagectomy specimens. Invasion in the upper third of the submucosa (sm1) is defined as up to 500 μm below the deepest fiber of the original muscularis mucosa, invasion into the middle third of the submucosa (sm2) is defined as up to 1000 μm depth, and finally invasion in the deeper third of the submucosa (sm3) as more than 1000 μm. The vertical safety margin (VM) from the deepest point of infiltration to the basal resection margin is measured in microns.

Resection Margins

The assessment of a specimen's margins is an equally complicated matter. This is because formal definitions of negative endoscopic resection margins have not been given. A positive lateral margin after local endoscopic resection is usually a minor clinical issue, because endoscopic resection will in most cases be accompanied by endoscopic ablation therapy. For this reason a minimal lateral safety margin (LM) has not been defined for the lateral margin. A positive deep (i.e., vertical) margin in pT1b disease correlates with a worse outcome and is a definitive indication for surgical esophagectomy. In many other instances in surgical pathology, it has been shown that a safety margin of <1 mm is associated with a greater risk of recurrence and is considered an R1 resection. In endoscopic resections, however, a minimal deep safety margin has not been defined. This is clearly an area for future research.

Artifacts

The detailed assessment of infiltration depth and deep margins can be further hampered by artifacts due to injection of fluid during the endoscopic procedure, poor handling and processing of the resection specimen, and shrinkage of the tissue following formalin fixation. Needle tracks can occur at the lateral border and cause artifacts that may mimic a positive basal resection margin. The lateral border of the specimen may retract and fold over during fixation, which may provoke concerns about the deep margin of the resection specimen (Fig. 8.7). Additional immunohistochemical stainings, such as a desmin stain to highlight muscle fibers, are an indispensable aid to visualize the (duplicated) muscularis mucosae in difficult cases (Fig. 8.7b). Given these issues, one should avoid measuring either the maximal infiltration depth or the R-status of the basal resection margin at the lateral border of an EMR specimen.

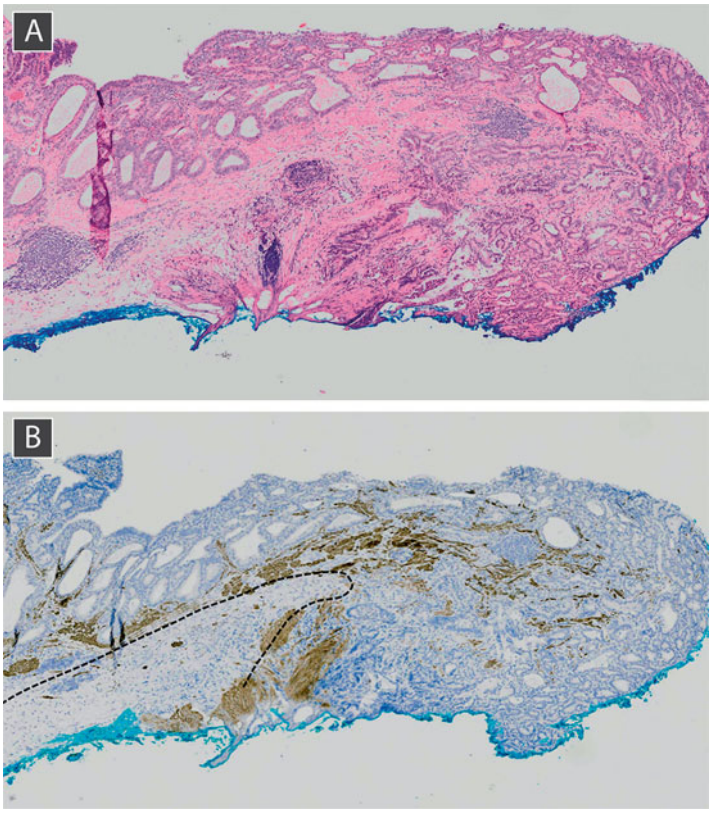

Fig. 8.7 (**a**) Folding over of the lateral edge of a mucosectomy specimen, possibly leading to overdiagnosis as a positive basal resection margin. (**b**) Desmin stain of figure (**a**), highlighting the folded contours of the muscularis mucosae (*dotted line*)

(Lympho-)Vascular Invasion

Vascular invasion or vessel permeation can involve small vessels like capillaries or lymphatic vessels, but also larger veins and arteries, although this occurs less frequently [42]. Its presence clearly portends a worse prognosis. Optional items to be recorded in the pathology report are the width of tumor infiltration in the submucosa, perineural invasion, and the total tumor thickness measured from the surface down to the deepest point of infiltration in microns.

Risk of Local Recurrence

Tumor differentiation grade, depth of invasion, basal margin status, and presence of vascular invasion are assessed to gauge recurrence risk and, by extension, the need for subsequent surgical esophagectomy. The main goal of esophagectomy in T1

adenocarcinoma is to attain local control in case of a positive basal margin in an endoscopic resection specimen and to curtail local recurrence after lymph node metastasis. Series reporting LNM in T1 esophageal adenocarcinoma vary from 16 to 44 % [39, 43–48], most series showing a lower rate of lymph node metastases in sm1 adenocarcinoma (0–20 %) as compared to deeper infiltrating lesions. However, these series are based on retrospective studies of surgical resection specimens, which may not have been as thoroughly investigated as EMR specimens. Manner et al. prospectively studied 72 patients with endoscopically resected sm1 (T1b) adenocarcinoma (mean follow-up of 60 months). In this study patients were stratified into low-risk (LR) ($n=49$) and high-risk (HR) ($n=23$) groups depending on the presence of ≥1 risk factor [37]. In the HR group, 9 % of patients developed LNM, while in the LR group only 2 % of patients developed LNM. While these data clearly support the current risk stratification scheme, they also suggest that many patients with early submucosal T1b cancers can be adequately and safely treated endoscopically. It is not clear which (combination) of the individual risk factors has the highest prognostic value for recurrence of disease. Further research of larger series with longer follow-up is necessary and will likely be combined with immunohistochemical and genetic profiling. For the time being, however, controversy in endoscopic treatment of sm1 submucosal cancers remains. Management of these patients should not take place outside of specialized centers.

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References

1. American Gastroenterological Association, Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association medical position statement on the management of Barrett's esophagus. Gastroenterology. 2011;140(3):1084–91. doi:10.1053/j.gastro.2011.01.030.
2. Fitzgerald RC, di Pietro M, Ragunath K, Ang Y, Kang JY, Watson P, Trudgill N, Patel P, Kaye PV, Sanders S, O'Donovan M, Bird-Lieberman E, Bhandari P, Jankowski JA, Attwood S, Parsons SL, Loft D, Lagergren J, Moayyedi P, Lyratzopoulos G, de Caestecker J, British Society of Gastroenterology. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. Gut. 2014;63(1):7–42. doi:10.1136/gutjnl-2013-305372.
3. Bennett C, Moayyedi P, Corley DA, DeCaestecker J, Falck-Ytter Y, Falk G, Vakil N, Sanders S, Vieth M, Inadomi J, Aldulaimi D, Ho KY, Odze R, Meltzer SJ, Quigley E, Gittens S, Watson P, Zaninotto G, Iyer PG, Alexandre L, Ang Y, Callaghan J, Harrison R, Singh R, Bhandari P, Bisschops R, Geramizadeh B, Kaye P, Krishnadath S, Fennerty MB, Manner H, Nason KS, Pech O, Konda V, Ragunath K, Rahman I, Romero Y, Sampliner R, Siersema PD, Tack J, Tham TC, Trudgill N, Weinberg DS, Wang J, Wang K, Wong JY, Attwood S, Malfertheiner P, MacDonald D, Barr H, Ferguson MK, Jankowski J. BOB CAT: a large-scale review and delphi consensus for management of Barrett's esophagus with no dysplasia, indefinite for, or low-grade dysplasia. Am J Gastroenterol. 2015;110(5):662–82. doi:10.1038/ajg.2015.55. quiz 683.

4. Riddell RH, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC, et al. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. Hum Pathol. 1983;14(11):931–68.
5. Schlemper R, Riddell R, Kato Y, Borchard F, Cooper H, Dawsey S, Dixon M, Fenoglio-Preiser C, Fléjou J-F, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim Y, Kirchner T, Klimpfinger M, Koike M, Lauwers G, Lewin K, Oberhuber G, Offner F, Price A, Rubio C, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. Gut. 2000;47(2):251–5. doi:10.1136/gut.47.2.251.
6. Voltaggio L, Montgomery E, Lam-Himlin D. A clinical and histopathologic focus on Barrett esophagus and Barrett-related dysplasia. Arch Pathol Lab Med. 2011;135:1249–60. doi:10.5858/arpa.2011-0019-RA.
7. Reid BJ, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H, et al. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. Hum Pathol. 1988;19(2):166–78.
8. Montgomery E, Bronner MP, Goldblum JR, Greenson JK, Haber MM, Hart J, Lamps LW, Lauwers GY, Lazenby AJ, Lewin DN, Robert ME, Toledano AY, Shyr Y, Washington K. Reproducibility of the diagnosis of dysplasia in Barrett esophagus: a reaffirmation. Hum Pathol. 2001;32(4):368–78. doi:10.1053/hupa.2001.23510.
9. Mahajan D, Bennett AE, Liu X, Bena J, Bronner MP. Grading of gastric foveolar-type dysplasia in Barrett's esophagus. Mod Pathol. 2010;23(1):1–11. doi:10.1038/modpathol.2009.147.
10. Odze RD. Diagnosis and grading of dysplasia in Barrett's oesophagus. J Clin Pathol. 2006;59(10):1029–38. doi:10.1136/jcp.2005.035337.
11. Horvath B, Singh P, Xie H, Thota PN, Allende DS, Pai RK, Patil DT, Plesec TP, Goldblum JR, Liu X. Risk for esophageal neoplasia in Barrett's esophagus patients with mucosal changes indefinite for dysplasia. J Gastroenterol Hepatol. 2015;30(2):262–7. doi:10.1111/jgh.12696.
12. Sonwalkar SA, Rotimi O, Scott N, Verghese E, Dixon M, Axon AT, Everett SM. A study of indefinite for dysplasia in Barrett's oesophagus: reproducibility of diagnosis, clinical outcomes and predicting progression with AMACR (alpha-methylacyl-CoA-racemase). Histopathology. 2010;56(7):900–7. doi:10.1111/j.1365-2559.2010.03571.x.
13. Younes M, Ertan A, Lechago LV, Somoano JR, Lechago J. p53 Protein accumulation is a specific marker of malignant potential in Barrett's metaplasia. Dig Dis Sci. 1997;42(4):697–701.
14. Lomo LC, Blount PL, Sanchez CA, Li X, Galipeau PC, Cowan DS, Ayub K, Rabinovitch PS, Reid BJ, Odze RD. Crypt dysplasia with surface maturation: a clinical, pathologic, and molecular study of a Barrett's esophagus cohort. Am J Surg Pathol. 2006;30(4):423–35.
15. Kaye PV, Haider SA, Ilyas M, James PD, Soomro I, Faisal W, Catton J, Parsons SL, Ragunath K. Barrett's dysplasia and the Vienna classification: reproducibility, prediction of progression and impact of consensus reporting and p53 immunohistochemistry. Histopathology. 2009;54(6):699–712. doi:10.1111/j.1365-2559.2009.03288.x.
16. Weaver JM, Ross-Innes CS, Shannon N, Lynch AG, Forshew T, Barbera M, Murtaza M, Ong CA, Lao-Sirieix P, Dunning MJ, Smith L, Smith ML, Anderson CL, Carvalho B, O'Donovan M, Underwood TJ, May AP, Grehan N, Hardwick R, Davies J, Oloumi A, Aparicio S, Caldas C, Eldridge MD, Edwards PA, Rosenfeld N, Tavare S, Fitzgerald RC, the OC. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet. 2014. doi:10.1038/ng.3013.
17. Menezes A, Tierney A, Yang YX, Forde KA, Bewtra M, Metz D, Ginsberg GG, Falk GW. Adherence to the 2011 American Gastroenterological Association medical position statement for the diagnosis and management of Barrett's esophagus. Dis Esophagus. 2014. doi:10.1111/dote.12228.
18. de Jonge PJ, van Blankenstein M, Grady WM, Kuipers EJ. Barrett's oesophagus: epidemiology, cancer risk and implications for management. Gut. 2014;63(1):191–202. doi:10.1136/gutjnl-2013-305490.
19. Timmer MR, Sun G, Gorospe EC, Leggett CL, Lutzke L, Krishnadath KK, Wang KK. Predictive biomarkers for Barrett's esophagus: so near and yet so far. Dis Esophagus. 2013;26(6):574–81. doi:10.1111/dote.12015.

20. Varghese S, Lao-Sirieix P, Fitzgerald RC. Identification and clinical implementation of biomarkers for Barrett's esophagus. Gastroenterology. 2012;142(3):435–41. doi:10.1053/j.gastro.2012.01.013. e432.
21. Wani S, Falk GW, Post J, Yerian L, Hall M, Wang A, Gupta N, Gaddam S, Singh M, Singh V, Chuang KY, Boolchand V, Gavini H, Kuczynski J, Sud P, Bansal A, Rastogi A, Mathur SC, Young P, Cash B, Goldblum J, Lieberman DA, Sampliner RE, Sharma P. Risk factors for progression of low-grade dysplasia in patients with Barrett's esophagus. Gastroenterology. 2011;141(4):1179–86. doi:10.1053/j.gastro.2011.06.055. 1186e1171.
22. Skacel M, Petras RE, Gramlich TL, Sigel JE, Richter JE, Goldblum JR. The diagnosis of low-grade dysplasia in Barrett's esophagus and its implications for disease progression. Am J Gastroenterol. 2000;95(12):3383–7. doi:10.1111/j.1572-0241.2000.03348.x.
23. Coco DP, Goldblum JR, Hornick JL, Lauwers GY, Montgomery E, Srivastava A, Wang H, Odze RD. Interobserver variability in the diagnosis of crypt dysplasia in Barrett esophagus. Am J Surg Pathol. 2011;35(1):45–54. doi:10.1097/PAS.0b013e3181ffdd14.
24. Kerkhof M, van Dekken H, Steyerberg EW, Meijer GA, Mulder AH, de Bruine A, Driessen A, ten Kate FJ, Kusters JG, Kuipers EJ, Siersema PD, Group Cs. Grading of dysplasia in Barrett's oesophagus: substantial interobserver variation between general and gastrointestinal pathologists. Histopathology. 2007;50(7):920–7. doi:10.1111/j.1365-2559.2007.02706.x.
25. Wani S, Mathur SC, Curvers WL, Singh V, Alvarez Herrero L, Hall SB, Ulusarac O, Cherian R, McGregor DH, Bansal A, Rastogi A, Ahmed B, Singh M, Gaddam S, Ten Kate FJ, Bergman J, Sharma P. Greater interobserver agreement by endoscopic mucosal resection than biopsy samples in Barrett's dysplasia. Clin Gastroenterol Hepatol. 2010;8(9):783–8. doi:10.1016/j.cgh.2010.04.028.
26. Curvers WL, ten Kate FJ, Krishnadath KK, Visser M, Elzer B, Baak LC, Bohmer C, Mallant-Hent RC, van Oijen A, Naber AH, Scholten P, Busch OR, Blaauwgeers HG, Meijer GA, Bergman JJ. Low-grade dysplasia in Barrett's esophagus: overdiagnosed and underestimated. Am J Gastroenterol. 2010;105(7):1523–30. doi:10.1038/ajg.2010.171.
27. Duits LC, Phoa KN, Curvers WL, Ten Kate FJ, Meijer GA, Seldenrijk CA, Offerhaus GJ, Visser M, Meijer SL, Krishnadath KK, Tijssen JG, Mallant-Hent RC, Bergman JJ. Barrett's oesophagus patients with low-grade dysplasia can be accurately risk-stratified after histological review by an expert pathology panel. Gut. 2014. doi:10.1136/gutjnl-2014-307278.
28. Lim CH, Treanor D, Dixon MF, Axon AT. Low-grade dysplasia in Barrett's esophagus has a high risk of progression. Endoscopy. 2007;39(7):581–7. doi:10.1055/s-2007-966592.
29. Pech O, Vieth M, Schmitz D, Gossner L, May A, Seitz G, Stolte M, Ell C. Conclusions from the histological diagnosis of low-grade intraepithelial neoplasia in Barrett's oesophagus. Scand J Gastroenterol. 2007;42(6):682–8. doi:10.1080/00365520601075803.
30. Vieth M, Schubert B, Lang-Schwarz K, Stolte M. Frequency of Barrett's neoplasia after initial negative endoscopy with biopsy: a long-term histopathological follow-up study. Endoscopy. 2006;38(12):1201–5. doi:10.1055/s-2006-944993.
31. Jung KW, Talley NJ, Romero Y, Katzka DA, Schleck CD, Zinsmeister AR, Dunagan KT, Lutzke LS, Wu TT, Wang KK, Frederickson M, Geno DM, Locke GR, Prasad GA. Epidemiology and natural history of intestinal metaplasia of the gastroesophageal junction and Barrett's esophagus: a population-based study. Am J Gastroenterol. 2011;106(8):1447–55. doi:10.1038/ajg.2011.130. quiz 1456.
32. Whiteman DC, Appleyard M, Bahin FF, Bobryshev YV, Bourke MJ, Brown I, Chung A, Clouston A, Dickins E, Emery J, Eslick GD, Gordon LG, Grimpen F, Hebbard G, Holliday L, Hourigan L, Kendall BJ, Lee EY, Levert A, Lord RV, Lord SJ, Maule D, Moss A, Norton I, Olver I, Pavey D, Raftopoulos S, Rajendra S, Schoeman M, Singh R, Sitas F, Smithers BM, Taylor A, Thomas ML, Thomson I, To H, von Dincklage J, Vuletich C, Watson DI, Yusoff IF. Australian clinical practice guidelines for the diagnosis and management of Barrett's esophagus and early esophageal adenocarcinoma. J Gastroenterol Hepatol. 2015. doi:10.1111/jgh.12913.
33. Vieth M, Riddell RH, Montgomery EA. High-grade dysplasia versus carcinoma: east is east and west is west, but does it need to be that way? Am J Surg Pathol. 2014;38(11):1453–6. doi:10.1097/PAS.0000000000000288.

34. Mino-Kenudson M, Hull MJ, Brown I, Muzikansky A, Srivastava A, Glickman J, Park DY, Zuckerberg L, Misdraji J, Odze RD, Lauwers GY. EMR for Barrett's esophagus-related superficial neoplasms offers better diagnostic reproducibility than mucosal biopsy. Gastrointest Endosc. 2007;66(4):660–6. doi:10.1016/j.gie.2007.02.063. quiz 767, 769.
35. Vieth M, Langner C, Neumann H, Takubo K. Barrett's esophagus. Practical issues for daily routine diagnosis. Pathol Res Pract. 2012;208(5):261–8. doi:10.1016/j.prp.2012.03.001.
36. Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO classification of tumours of the digestive system, WHO series on histological and genetic typing of human tumours, vol. 3. Geneva: World Health Organization; 2010.
37. Manner H, Pech O, Heldmann Y, May A, Pauthner M, Lorenz D, Fisseler-Eckhoff A, Stolte M, Vieth M, Ell C. The frequency of lymph node metastasis in early-stage adenocarcinoma of the esophagus with incipient submucosal invasion (pT1b sm1) depending on histological risk patterns. Surg Endosc. 2015;29(7):1888–96. doi:10.1007/s00464-014-3881-3.
38. Holscher AH, Vallbohmer D, Bollschweiler E. Early Barrett's carcinoma of the esophagus. Ann Thorac Cardiovasc Surg. 2008;14(6):347–54.
39. Westerterp M, Koppert LB, Buskens CJ, Tilanus HW, ten Kate FJ, Bergman JJ, Siersema PD, van Dekken H, van Lanschot JJ. Outcome of surgical treatment for early adenocarcinoma of the esophagus or gastro-esophageal junction. Virchows Arch. 2005;446(5):497–504. doi:10.1007/s00428-005-1243-1.
40. Vieth M, Stolte M. Pathology of early upper GI cancers. Best Pract Res Clin Gastroenterol. 2005;19(6):857–69. doi:10.1016/j.bpg.2005.02.008.
41. Kaneshiro DK, Post JC, Rybicki L, Rice TW, Goldblum JR. Clinical significance of the duplicated muscularis mucosae in Barrett esophagus-related superficial adenocarcinoma. Am J Surg Pathol. 2011;35(5):697–700. doi:10.1097/PAS.0b013e3182159c4b.
42. Kumarasinghe MP, Brown I, Raftopoulos S, Bourke MJ, Charlton A, de Boer WB, Eckstein R, Epari K, Gill AJ, Lam AK, Price T, Streutker C, Lauwers GY. Standardised reporting protocol for endoscopic resection for Barrett oesophagus associated neoplasia: expert consensus recommendations. Pathology. 2014;46(6):473–80. doi:10.1097/PAT.0000000000000160.
43. Stein HJ, Feith M, Bruecher BLDM, Naehrig J, Sarbia M, Siewert JR. Early esophageal cancer—pattern of lymphatic spread and prognostic factors for long-term survival after surgical resection. Ann Surg. 2005;242(4):566–75. doi:10.1097/01.sla.0000184211.75970.85.
44. Liu L, Hofstetter WL, Rashid A, Swisher SG, Correa AM, Ajani JA, Hamilton SR, Wu TT. Significance of the depth of tumor invasion and lymph node metastasis in superficially invasive (T1) esophageal adenocarcinoma. Am J Surg Pathol. 2005;29(8):1079–85.
45. Bollschweiler E, Baldus SE, Schroder W, Prenzel K, Gutschow C, Schneider PM, Holscher AH. High rate of lymph-node metastasis in submucosal esophageal squamous-cell carcinomas and adenocarcinomas. Endoscopy. 2006;38(2):149–56. doi:10.1055/s-2006-924993.
46. Ancona E, Rampado S, Cassaro M, Battaglia G, Ruol A, Castoro C, Portale G, Cavallin F, Rugge M. Prediction of lymph node status in superficial esophageal carcinoma. Ann Surg Oncol. 2008;15(11):3278–88. doi:10.1245/s10434-008-0065-1.
47. Hagen JA, DeMeester SR, Peters JH, Chandrasoma P, DeMeester TR. Curative resection for esophageal adenocarcinoma: analysis of 100 en bloc esophagectomies. Ann Surg. 2001;234(4):520–30. discussion 530–521.
48. Rice TW, Zuccaro Jr G, Adelstein DJ, Rybicki LA, Blackstone EH, Goldblum JR. Esophageal carcinoma: depth of tumor invasion is predictive of regional lymph node status. Ann Thorac Surg. 1998;65(3):787–92.

Chapter 9
Staging Early Esophageal Cancer

O.J. Old, M. Isabelle, and H. Barr

Principles of Staging

Cancer staging provides a measure of disease burden in the patient, through assessment of local tumor invasion, histological tumor grade, nodal and metastatic disease. This information is essential to plan the appropriate treatment options for a given tumor in each patient and is a useful guide to prognosis. Consequently, accurate staging is of great importance. In esophageal cancer the depth of local invasion, extent of nodal disease, and the presence or absence of metastatic disease will all determine the optimal treatment for a patient. One of the key issues for early esophageal cancer is depth of invasion: more superficial lesions confined to the mucosa are amenable to curative endoscopic resection, both because complete excision margins may be more readily achieved and because of the low risk of associated nodal metastasis. In the presence of deeper invasion or nodal disease, local treatment with endoscopic resection will be insufficient therapy, and surgical resection (along with any adjuvant therapies) will be required to achieve a cure. Assessment of the presence and extent of lymph node metastasis is useful for determining the likelihood of achieving regional control [1]. Detection of more distant

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metastatic spread is crucial in identifying those who will not benefit from surgery and for whom surgical resection would be futile. This information also enables the appropriate treatment pathway to be instituted earlier, whether that may be palliative chemotherapy or achieving optimal symptom control. The widespread use of neoadjuvant therapy in esophageal cancer has resulted in frequent restaging after initial therapy, and therefore accurate staging is not just important to decide treatment options but can be used to assess response to therapy and guide the next stages of treatment.

Clinical staging aims to determine the extent of the tumor prior to treatment, and combines information from clinical examination, clinical procedures such as endoscopy or staging laparoscopy, laboratory studies, and imaging techniques. Clinical staging is not necessarily a "one-off" assessment, but may be repeated and revised with further investigations, for example, to monitor the effects of therapy, or if there are concerns about tumor progression. For esophageal cancer, clinical staging will begin with history and examination of the patient, since in some cases signs of metastatic disease may be present at this stage. Endoscopy is now the first-line investigation if esophageal neoplasia is suspected, and this has replaced barium swallow: endoscopy offers superior characterization of any lesion, offers the possibility of biopsy, and the potential for endoscopic therapy. If malignancy is confirmed at endoscopy and biopsy then imaging studies will be performed to characterize further the extent of disease—these are discussed further as follows.

Histopathological staging involves analysis of tissue specimens, whether from biopsy, endoscopic resection, or surgical resection of the entire specimen and en bloc lymph nodes. Biopsy taken at endoscopy usually provides the initial tissue diagnosis to confirm malignancy. As with clinical staging, this may be revised if further tissue is received, for example, if an endoscopic resection is followed by an esophagectomy. Complete staging combines information from both clinical and histopathological staging. Once a diagnosis of cancer has been made, the case should be discussed at a multidisciplinary team meeting with specialist input from the surgical team, pathology, radiology, oncology, dieticians, and specialist nurses. Decisions about further investigation and treatment should then be made with the patient, taking account of the specialist MDT opinion (Figs. 9.1 and 9.2).

TNM Staging System

The most widely used classification system for esophageal cancers, which reports the stage in terms of primary tumor (T), nodal spread (N), and distant metastases (M), is the TNM staging system published jointly by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC). The 7th edition, published in 2009, was developed based on data gathered from 13 institutions and 4627 patients by the World Esophageal Cancer Collaboration, to incorporate prognostic significance into the various stage groupings [2]. Consequently, there are a number of changes from the 6th edition [3]. T_{is} now

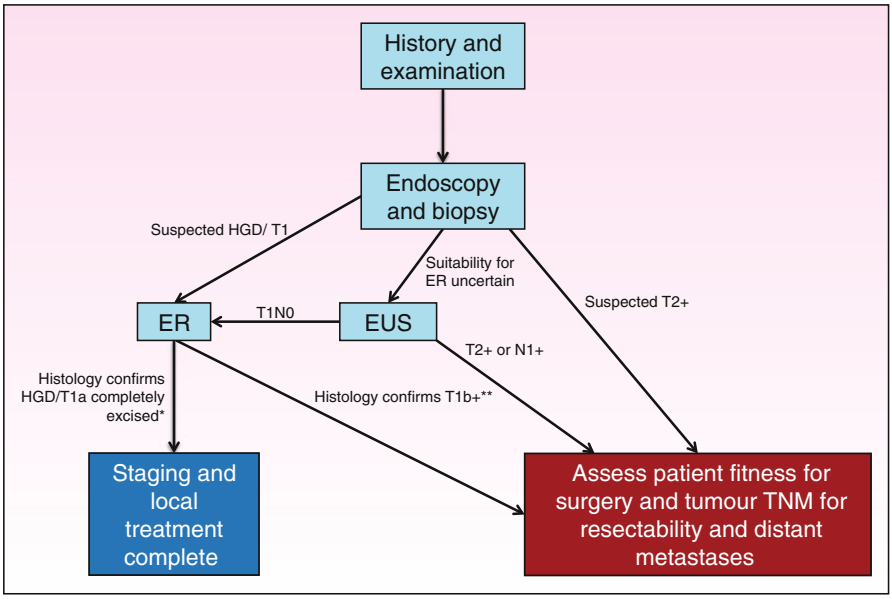

Fig. 9.1 Algorithm for staging early esophageal cancer. *If histology confirms T1a but incompletely excised, further ER may be considered or alternatively assessment for surgical resection may be preferred. **T1bsm1 may be considered as complete therapy for high-risk surgical candidates (see Fig. 9.2). *ER* endoscopic resection, *EUS* endoscopic ultrasound, *HGD* high grade dysplasia

includes high-grade dysplasia or any noninvasive neoplastic epithelium; the T4 category has now been subdivided based on whether the locally invaded structures are resectable (T4a) or unresectable (T4b); the number of positive lymph nodes is taken into account to subdivide into N0–N3, and cervical and celiac lymph nodes are considered regional lymph nodes along with other paraesophageal nodes; consequently, the M1a categories (previously designating cervical or celiac lymph nodes) have now been lost, and M1b now becomes M1. Stage groupings of adenocarcinoma and squamous cell carcinoma are no longer equivalent, and staging of esophagogastric junctional tumors is now harmonized, where previously classification depended on use of esophageal or gastric groupings [3]. The TNM 7th edition is shown in the following tables (Tables 9.1, 9.2, and 9.3).

The TNM stage may be determined by either clinical or pathological staging: the prefix "c" denotes clinical staging, and the prefix "p" indicates pathological staging. If neoadjuvant treatment has been given prior to surgical excision and pathological staging, an additional "y" prefix is added, e.g., ypT2N1M0 [4].

In recognition of the clinical significance of invasion of certain layers of the submucosa, there is further subdivision for early cancers, and the following terms may be used in the context of early esophageal cancer: "intramucosal carcinoma" (IMC) is equivalent to T1a, and "Submucosal invasion by adenocarcinoma" for any esophageal adenocarcinoma that has invaded into the submucosa (T1b) [5]. Further classi-

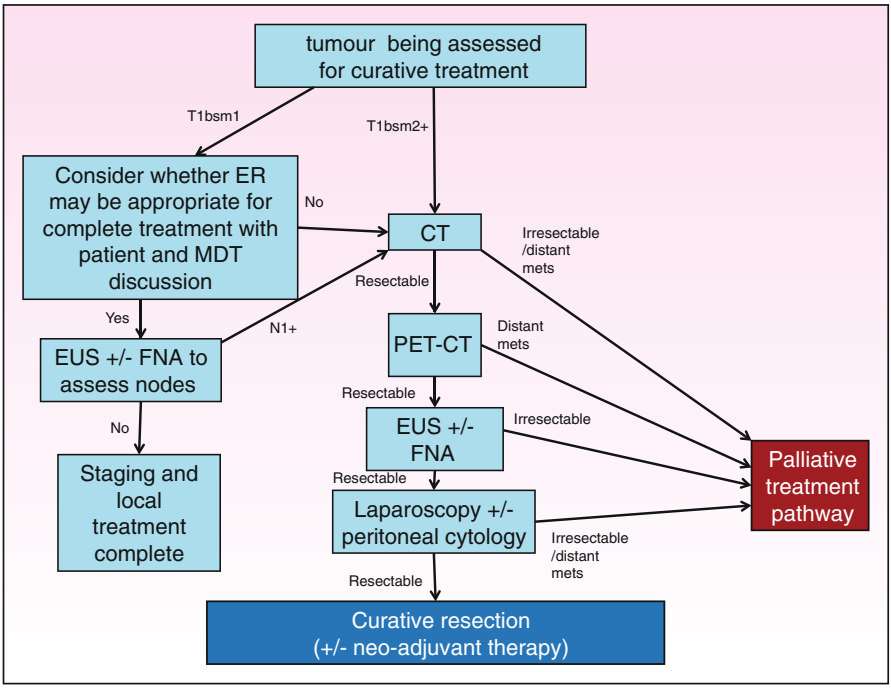

Fig. 9.2 Algorithm for staging advanced esophageal cancer. *ER* endoscopic resection, *MDT* multidisciplinary team, *EUS* endoscopic ultrasound, *FNA* fine needle aspiration, *CT* computed tomography, *PET-CT* positron emission tomography-CT, *Mets* metastases

fication is based on the depth of invasion of either the mucosa (m1-4) or submucosa (sm1-3). The clinical significance lies in the risk of lymph node metastasis associated with deeper invasion. When confined to the mucosa, risk of nodal metastasis is below 3 % [6–13], and consequently local treatment with endoscopic resection (ER) has resulted in very good tumor free and overall survival in high-volume centers. One recent large series of ERs for T1a cancer reported tumor-free survival of 93.8 % at mean follow-up 56.6 months [14]. Once the submucosa has been invaded the risk of nodal involvement rises (up to 46 %), and therefore ER for T1b tumors is associated with poorer outcomes, with 5-year tumor free and overall survival 60 and 58 %, respectively [6–12, 15]. For tumors within the upper third of the submucosa (T1bsm1), the risk of lymph node metastasis remains relatively low (around 10 %) and some series report good outcomes with ER [7, 11, 14]. Deciding whether treatment is complete in these cases may be further influenced by pathological indicators of good prognosis, such as R0 resection and absence of vascular and lymphatic invasion [16]. The use of ER for T1bsm1 cancer remains debated however, and the current British Society of Gastroenterology (BSG) guidance advises surgery for patients who are fit enough, but that ER should be offered with curative intent in patients who are high-risk surgical candidates [5].

Table 9.1 UICC/AJCC tumor-node-metastasis (TNM) staging system for esophageal cancer (7th edition) adapted with permission from Ref. [60]

T status	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	High grade dysplasia
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures
T4a	Resectable tumor invading the pleura, pericardium or diaphragm.
T4b	Unresectable tumor invading other adjacent structures such as the aorta, vertebral body, or trachea
N status	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–2 regional lymph nodes
N2	Metastasis in 3–6 regional lymph nodes
N3	Metastasis in 7 or more regional lymph nodes
M status	
Mx	Distant metastases cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
Histological grading	
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Endoscopic Resection (ER)

ER is recommended as the treatment of choice for visible dysplastic lesions and T1a cancer, and while it should be performed with therapeutic intent, it is also established as the most accurate staging modality for assessing depth of invasion in early neoplasia [5]. It should be noted that the term "endoscopic resection" (ER) is now preferred to the previous nomenclature which referred to "endoscopic mucosal resection" (EMR) (to make the distinction from "endoscopic submucosal dissection"): it is recognized that endoscopic resection is not necessarily limited to the mucosa and indeed will give the most accurate staging information if some of the submucosa is included in the resection specimen. A number of studies have investigated the value of ER as a staging tool and its ability to alter diagnosis and influence patient management. There is wide variation in the reported frequency

Table 9.2 UICC/AJCC 7th edition esophageal adenocarcinoma stage groupings (reproduced with permission from Ref. [2])

Stage	T	N	M	G
0	Is (HGD)	0	0	1
IA	1	0	0	1–2
IB	1	0	0	3
	2	0	0	1–2
IIA	2	0	0	3
IIB	3	0	0	Any
	1–2	1	0	Any
IIIA	1–2	2	0	Any
	3	1	0	Any
	4a	0	0	Any
IIIB	3	2	0	Any
IIIC	4a	1–2	0	Any
	4b	Any	0	Any
	Any	N3	0	Any
IV	Any	Any	1	Any

Table 9.3 UICC/AJCC 7th edition esophageal squamous cell carcinoma stage groupings (reproduced with permission from Ref. [2])

Stage	T	N	M	G	Location
0	Is (HGD)	0	0	1	Any
IA	1	0	0	1	Any
IB	1	0	0	2–3	Any
	2–3	0	0	1	Lower
IIA	2–3	0	0	1	Upper, middle
	2–3	0	0	2–3	Lower
IIB	2–3	0	0	2–3	Upper, middle
	1–2	1	0	Any	Any
IIIA	1–2	2	0	Any	Any
	3	1	0	Any	Any
	4a	0	0	Any	Any
IIIB	3	2	0	Any	Any
IIIC	4a	1–2	0	Any	Any
	4b	Any	0	Any	Any
	Any	N3	0	Any	Any
IV	Any	Any	1	Any	Any

with which ER changes the preprocedure histological diagnosis, varying from 25 to 70 % [17–21], but the largest single study containing data from 293 ER procedures found that ER altered the histology in 49 % of cases, and changed management decisions in 30 % [21].

ER of visible lesions not only gives accurate information about depth at the resected tissue site, but is also likely to identify the most advanced disease present in the patient: data from stepwise ER of entire Barrett's segments have confirmed that the most advanced disease is located in visible lesions [22]. A meta-analysis of studies in which patients have undergone esophagectomy for HGD found the risk of invasive OAC to be 11 % in those with visible lesions, compared to 3 % in those with no visible lesion [23].

For early Barrett's neoplasia, where the lesion is clinically suspected to be HGD/T1 only, current guidelines advocate proceeding directly to ER, with no evidence to support prior investigation with CT or PET-CT [5]. While these modalities are useful in identifying distant metastases, they are poor at assessing depth of local invasion (discussed further below). The guidelines acknowledge that in clinical practice there may be uncertainty over whether a lesion is HGD/T1, and hence suitable for ER, or a more advanced neoplasm. In these circumstances endoscopic ultrasound (EUS) is recommended to guide decision-making, although its limitations for superficial lesions are recognized, and its routine use prior to ER is not recommended.

If ER histology shows disease confined to the mucosa, with complete excision, no further staging is routinely indicated, although every case should be discussed at a specialist cancer multidisciplinary team meeting (MDT). If the lesion is invading the submucosa then surgery (plus consideration of neo-adjuvant therapy) should be offered to those fit enough, and further investigation is required to confirm the tumor is resectable and there are no distant metastases. For those patients with T1bsm1 lesions with good prognostic features (well differentiated and without lymphovascular invasion), ER may be considered as complete therapy [5]. However with the increased risk of nodal metastasis associated with submucosal invasion, current UK guidelines advise this approach only in those patients considered high-risk surgical candidates. Assessment of locoregional lymph nodes with EUS and fine-needle aspiration (FNA) may be helpful to inform this decision (see below), since if nodal disease is identified then ER will be insufficient to achieve local control.

Endoscopic Ultrasound (EUS)

EUS uses ultrasound to image the layers of the esophageal wall and gauge depth of invasion, and identify involved locoregional lymph nodes. EUS may be performed using a conventional echoendoscope or a high frequency miniprobe. Conventional echoendoscopes use 7.5 or 12 MHz frequency to image the five layers of the esophageal wall (see Table 9.4) [24]. A radial echoendoscope gives 360° view of the esophagus and surrounding mediastinum, but does not allow fine needle aspiration (FNA) to sample lymph nodes, which can be achieved with a linear echoendoscope. Tumors typically appear as a hypoechoic mass lesion disrupting the layers of the esophageal wall. Conventional EUS has an overall accuracy of 80–90 % for T-staging [25], but this varies with T-stage and is less accurate for early tumors [26]. EUS understages tumors in around 15–25 % of cases, and overstages lesions in 4–12 % compared against ER [26–29].

Table 9.4 Esophageal wall visualized at EUS with conventional echoendoscope (reproduced with permission from Ref. [24])

EUS layer	Esophageal wall layer	Echogenicity
1	Interface between lumen and mucosa	Hyperechoic
2	Deep mucosa including muscularis mucosa	Hypoechoic
3	Submucosa	Hyperechoic
4	Muscularis propria	Hypoechoic
5	Adventitia interface	Hyperechoic

Table 9.5 Esophageal wall visualized at EUS with high frequency miniprobe (reproduced with permission from Ref. [24])

EUS layer	Esophageal wall layer	Echogenicity
1	Interface between lumen and superficial mucosa	Hyperechoic
2	Interface between superficial and muscularis mucosa	Hypoechoic
3	Muscularis mucosa	Hyperechoic
4	Interface between muscularis mucosa and submucosa	Hypoechoic
5	Submucosa	Hyperechoic
6	Muscularis propria: inner circular muscle	Hypoechoic
7	Muscularis propria: intermuscular connective tissue	Hyperechoic
8	Muscularis propria: outer longitudinal muscle	Hypoechoic
9	Adventitia interface	Hyperechoic

Miniprobes use higher frequency ultrasound in the range 20–30 MHz, enabling greater detail to be imaged and visualization of the esophageal wall in nine layers (see Table 9.5) [24]. This greater detail is particularly useful for staging depth of early tumors (Fig. 9.3), which has been shown to be improved with high frequency miniprobes vs. conventional EUS [30], although even with the miniprobe, accurate T staging was achieved in only 64 % of cases in this series. Miniprobes also enable assessment of obstructing tumors that cannot be accessed by conventional echoendoscopes.

EUS also enables assessment of regional lymph nodes. In addition to the size of nodes, which can be seen on computed tomography (CT), EUS provides information on shape, echo intensity, and border demarcation. Benign lymph nodes are typically oval, elongated or triangular in shape, hyper- or isoechoic, and have poorly demarcated borders. Features suggesting malignancy (in decreasing order of importance) are as follows: size greater than 10 mm, round shape, well-demarcated borders, and hypoechoic structure [24, 31, 32]. While visual assessment can be subjective, the overall accuracy of these four visual criteria is around 75 % [33, 34], with one study reporting that if all four criteria are present, malignancy could be predicted with 100 % accuracy [31]. EUS assessment of lymph nodes is improved with the addition of EUS-guided FNA, with overall sensitivity, specificity, and accuracy for locoregional nodes around 90 % [35, 36]. When performing FNA care should be taken not to traverse the tumor, to avoid the risk of seeding malignancy from the primary, and

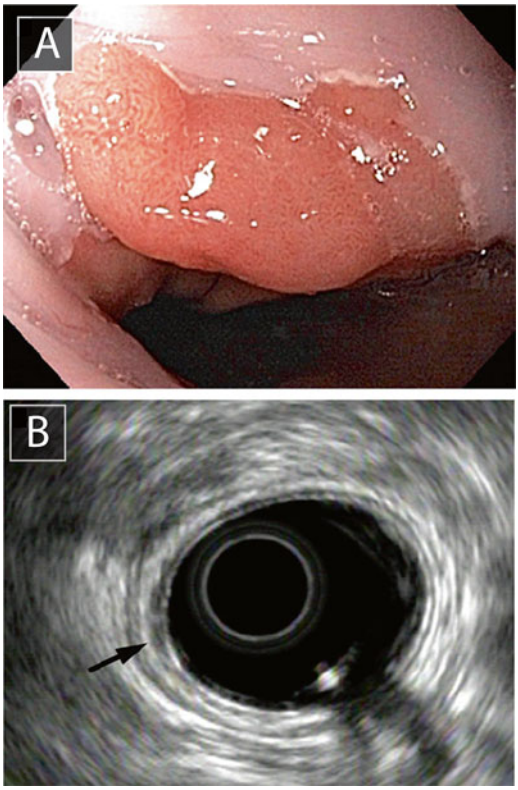

Fig. 9.3 Nodular lesion on a background of Barrett's esophagus seen at (**a**) endoscopic white light image, (**b**) EUS showing minimal wall thickening confined to the mucosa, without disruption of the submucosa (*arrow*). The lesion was excised at ER, and histology confirmed a T1a tumor. (reproduced with permission from Ref. [43])

to prevent false-positive results. EUS with FNA is the most accurate modality for assessing locoregional lymph nodes. A small study comparing EUS against CT and positron emission tomography (PET) found EUS gave accurate N staging in 81 % of patients, vs. 69 % for CT and 56 % for PET [37]. In a larger study directly comparing EUS-FNA with PET, PET did not alter nodal staging in any of the 148 patients undergoing both investigations [38].

For early neoplasia in Barrett's esophagus, EUS gives more accurate staging than CT: in a study of 100 consecutive patients with early esophageal cancer who underwent EUS and CT, CT did not change TNM staging in any cases and showed far lower sensitivity (38 % vs. 75 % for EUS) to identify nodal metastasis [39]. Nonetheless, because of the limitations in assessing depth of invasion in early esophageal cancer, there is debate as to whether EUS is adequate for determining whether endoluminal therapy is sufficient for these lesions [40–42]. Consequently, ER has replaced EUS in the staging of superficial lesions since it not only provides more accurate staging, but may simultaneously deliver curative treatment [24, 42]. For more advanced lesions, EUS provides accurate information on T-staging and regional lymph node involvement, and is an essential part of preoperative evaluation.

Computed Tomography (CT)

Once an esophageal cancer has been identified at endoscopy and biopsy, as a comparatively low cost and readily available imaging modality, CT is frequently the next investigation undertaken. CT can assess invasion into adjacent structures for T4 tumors and detect nodal and distant metastatic disease with CT of the chest, abdomen, and pelvis. Detection of distant metastasis on CT would indicate that a palliative treatment pathway was appropriate and render further investigations to assess resectability unnecessary. CT has a limited role in evaluating T-status. Tumors may appear as a focal or diffuse region of wall thickening, but sensitivity (for all T-grades) is low, with one study reporting sensitivity 67 % [34]. Unlike EUS, CT cannot resolve separate layers of the esophageal wall, limiting its ability to assess T-status [43]. However, CT can inform decisions on resectability where there is gross invasion into adjacent structures or loss of the periesophageal fat planes seen with tumor invasion or local fibrosis [43, 44]. When the periesophageal fat plane is intact invasion is highly unlikely, but the loss of the plane is not a highly specific finding [4].

CT assessment of nodal disease relies on size criteria, and consequently metastases within normal sized lymph nodes cannot be detected, reducing sensitivity, and lymph nodes may be enlarged due to other causes such as inflammation, reducing specificity. While differing size criteria may be used [4], in general intrathoracic and abdominal nodes are considered suspicious for malignancy if measuring greater than 10 mm in short axis, while supraclavicular nodes are suspicious if greater than 5 mm, and retrocrural nodes greater than 6 mm [43]. A meta-analysis of imaging performance in staging esophageal cancer found pooled sensitivity for CT to detect regional lymph nodes was 57 % and specificity 83 % [45]. For abdominal nodes the sensitivity was still lower at 42 %, with specificity 93 %.

The chief role of CT in staging esophageal cancer is identification of distant metastases. This is of great value in esophageal cancer where up to 30 % of patients present with metastatic disease [1], with the majority of (nonnodal) metastases to liver or lung [46]. The sensitivity of CT for detecting liver metastasis is 86–98 % [4]. A study by Lowe et al. (2005) of 75 patients comparing CT, PET, and EUS found the sensitivity and specificity of CT for identifying any distant metastasis was 81 and 82 %, respectively [47]. A meta-analysis by van Vliet et al. calculated pooled sensitivity of CT in detecting distant metastases was 52 % and specificity 91 % [45], though it should be noted that this included studies that defined nonregional lymph nodes as M1a (using the earlier TNM classification), and therefore the low sensitivity may partly be accounted for by the failure to identify positive nonregional nodes. Peritoneal spread of disease is easily missed on CT since peritoneal deposits are frequently small [4, 24]. Such deposits can be identified at laparoscopy however, and for this reason laparoscopy is now recommended as part of preoperative staging (discussed further later).

PET

Positron Emission Tomography (PET) is a functional imaging technique, which depends on physiological consumption of a radiolabeled agent. The most commonly used agent is a radiolabeled glucose analog, 18-fluorodeoxyglucose (^{18}F-FDG), which is concentrated in tissues with high glucose consumption, and on this basis can be used to identify malignant tissue [48]. Fused PET and CT imaging (PET-CT) offers advantages over PET alone, with the CT providing anatomical reference data and allowing images which combine anatomical and functional (metabolic) information [48]. Integrated PET-CT has greater accuracy for tumor staging than PET alone [49]. PET is comparatively poor at assessing T-status, and assessment of locoregional lymph nodes is limited by signal uptake from the adjacent tumor [50]. Consequently, PET is of limited value in early (T1) cancers. One study evaluated the use of PET-CT in patients with early esophageal cancer (cT1N0). In this study of 79 patients, PET-CT had sensitivity 0 % and positive predictive value 0 % for patients subsequently confirmed to have nodal disease on postoperative pathological staging, and the authors concluded that PET-CT in early esophageal cancer could be detrimental to patient care and lead to inappropriate treatment decisions [51].

The main role of PET is in identification of distant nodal and metastatic disease. PET has been reported to identify a clinically relevant change to disease stage in around 15 % of patients otherwise considered to have resectable disease based on EUS and CT staging alone [52, 53]. The study by Lowe et al. comparing multiple imaging modalities found PET had sensitivity 81 % and specificity 91 % for distant metastases [47]. The meta-analysis conducted by van Vliet et al. concluded that the sensitivity of PET for distant metastases was 71 %, with specificity 93 % (with the same caveats mentioned above). PET has also been shown to have a prognostic significance based on the maximum value of tumor metabolic activity (standardized uptake value, SUV), though whether this is independent of TNM staging is unclear [54].

Combined use of CT, EUS, and PET is recommended by current UK guidelines as each imaging modality contributes unique staging information for each patient [1, 47]. A combined approach also allows information from one study to aid interpretation of the others, e.g., using PET to assess an "indeterminate" nodule identified on CT, or using PET to guide FNA of possible nodal metastases when performing EUS. The results of these investigations can also be used to inform the need for other imaging modalities, e.g., if CT and PET are equivocal regarding a liver lesion, MRI may be used to characterize the lesion further. PET is also widely used in restaging patients following neo-adjuvant therapy. This ensures that there has not been significant tumor progression that would render the planned surgical resection futile, and also allows identification of responders and nonresponders to neo-adjuvant treatment, which can be used to plan further adjuvant therapy [55].

Staging Laparoscopy and Peritoneal Cytology

Both CT and PET are poor at identifying peritoneal disease, and may miss small liver lesions. If CT, PET, and EUS all indicate tumor resectability, then staging laparoscopy is performed, and if any such lesions are identified biopsies can be taken. Current UK guidelines advise staging laparoscopy for selected patients with lower esophageal or junctional tumors [1]. A number of studies have shown that staging laparoscopy can identify lesions in 10–20 % of patients undergoing assessment, thus avoiding futile surgery [56–58]. In the largest reported series, 416 patients with esophagogastric tumors assessed to be resectable underwent staging laparoscopy, with the authors reporting that laparoscopy altered management decisions for 20 % of patients. It should be noted however that only 11.5 % underwent both CT and EUS, and none of the patients had a PET scan [59].

Staging laparoscopy also allows sampling of peritoneal fluid to enable cytological analysis. If free fluid is present it should be sampled or peritoneal washings may be taken. While the presence of malignant ascites would indicate metastatic spread, the finding of free cancer cells in the absence of ascites is not clear. There is evidence, however, that these patients have a poor prognosis and this has led some to conclude that these patients would not benefit from resection with curative intent [58].

Future Staging Modalities: Optical Diagnosis

Optical Coherence Tomography (OCT)

OCT is analogous to ultrasound imaging but uses backscattering of near-infrared light to produce high-resolution high quality images (limited to 10 μm spatial resolution) of the gastrointestinal (GI) epithelial and subepithelial tissues with depths of 1–2 mm, generally restricting OCT imaging to the mucosa and submucosa when performed during endoscopy (Fig. 9.4). It is performed by using probes passed through the instrument channel of endoscopes to noninvasively capture the backscattered light from GI tissue, which can be later processed to create 3d images of the tissue morphology (Fig. 9.5) to determine the location of subsurface structures and any abnormalities [61, 62]. A number of studies have been published that have demonstrated the role of OCT in the detection of dysplasia [65–67]. One study involving 49 patients, demonstrated that OCT was able to diagnosis intramucosal carcinoma (IMC) and high-grade dysplasia (HGD) with a sensitivity and specificity of 83 and 75 %, respectively [66].

Other and some more recent studies [68–73] have demonstrated OCT in the identification and differentiation of Barrett's esophagus, high-grade dysplasia, and esophageal adenocarcinoma vs. the layered epithelial architecture in normal esophageal tissue, in which this layered epithelial tissue architecture becomes replaced with more heterogeneous tissue structures such as distorted and glandular

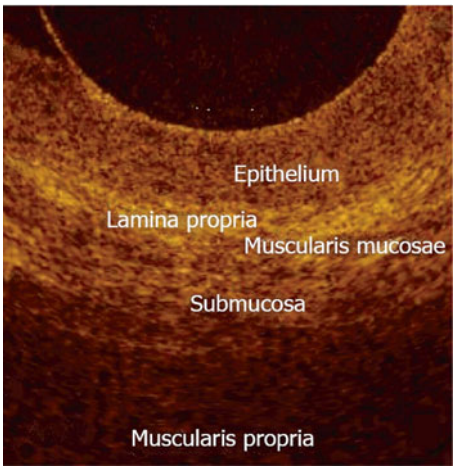

Fig. 9.4 Magnification of an OCT image showing normal esophageal wall. The OCT image shows a multiple-layer structure characterized by a superficial weakly scattering (hypo-reflective) layer, corresponding to the squamous epithelium, a highly scattering (hyper-reflective) layer corresponding to the lamina propria, a weakly scattering layer corresponding to the muscularis mucosae, difficult to recognize, a moderately scattering layer corresponding to the submucosa, and a weakly scattering, deep layer corresponding to muscularis propria. Reproduced with permission from Ref. [63]

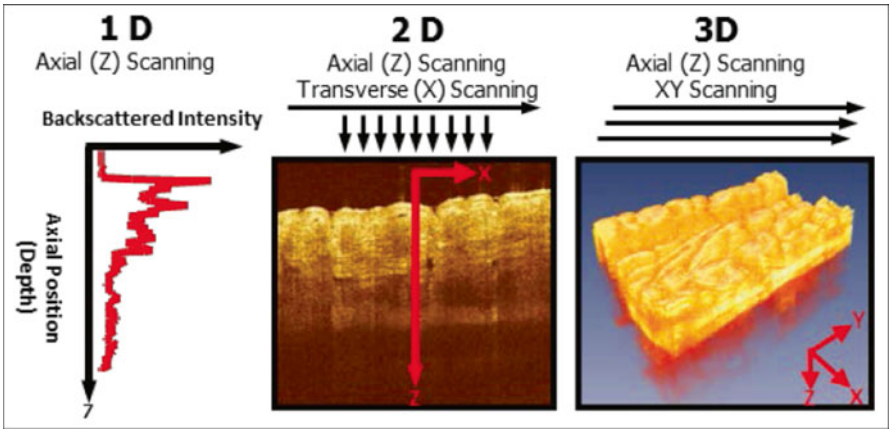

Fig. 9.5 Optical coherence tomography (OCT) generates cross-sectional and 3D images of tissue microstructure by measuring the echo time delay and magnitude of backscattered light. Architectural morphology can be imaged in vivo and in real time. Reproduced with permission from Ref. [64]

features, esophagitis edema, and fibrinoid deposits (Figs. 9.6 and 9.7). OCT has been used to identify the muscularis muscoae in ex vivo specimens and, as the authors mentioned, OCT could be used to delineate the esophageal mucosa layers and therefore identify the presence and depth of mucosa neoplasms [74].

Using OCT in preoperative staging of superficial esophageal squamous cell carcinomas (SESCCs), Hatta et al were to demonstrate that accuracy for epithelium (EP) or

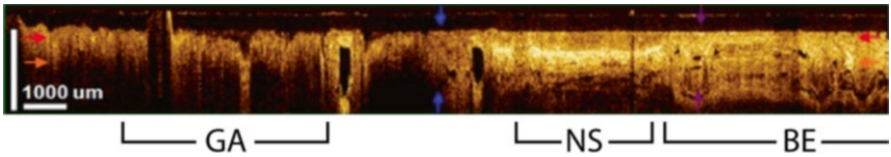

Fig. 9.6 Barrett's esophagus (BE) without dysplasia. Cross-sectional OCT imaging showing clear differences in layered architecture between gastric (GA), normal squamous (NS), and BE regions. BE regions exhibit distortion of the layered architecture and abnormal glandular features

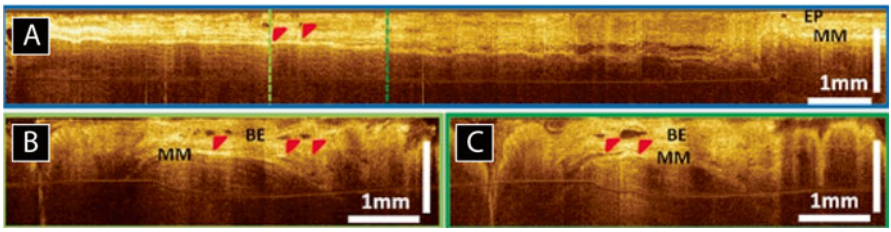

Fig. 9.7 Cross-sectional OCT images around GEJ. BE glands (*red arrows*) are clearly observed (*EP* epithelium, *MM* muscularis mucosae in photos **a**–**c**). Reproduced with permission from Ref. [81]

lamina propria mucosa (LPM) was significantly higher than that by using EUS (OCT, 94.6 %; HF-EUS, 80.6 %; $P<0.05$) [75] and in a similar study involving 62 patients, this group similarly showed that OCT was able to stage tumor infiltration of SESCCs for EP/LPM, muscularis mucosa (MM), submucosa (SM) with an accuracy rate of 94.9, 85, and 90.9 %, respectively. Additionally, the penetration depth of the OCT was enough to enable the depiction of the boundary of the deep regions of the tumor lesion [76]. However, this study demonstrated some limitations of OCT in its inability to identify cancer cell invasion and inflammatory cell infiltration.

Using OCT to identity and assess metastatic involvement in lymph nodes has been documented. In a 19 patient study, McLaughlin et al. [77] demonstrated the capability of OCT to image nodal microarchitecture through an assessment of fresh, unstained ex vivo lymph node samples with strong correlation between the OCT processed images and histology in 91 % of the lymph node tissue samples.

Due to limited depth of penetration, there is a limit to how far OCT could be used to interrogate the multiple-layer esophageal wall to determine the presence and depth of penetration of the mucosa neoplasms and lymph nodes tissue structure to identify the presence of micrometastases. Further technological advances are needed to improve both the spatial resolution and depth profiling of this type of tomography imaging [74]. Using more advanced version of OCT imaging, a number of groups [78–80] have shown that a needle-encased OCT probe could be used, interstitially, to collect OCT data from lymph nodes in vivo. Continued development of the probe-based endoscopy system and their inclusion in larger prospective clinical trials will determine the validity of OCT in the staging of early esophageal cancer.

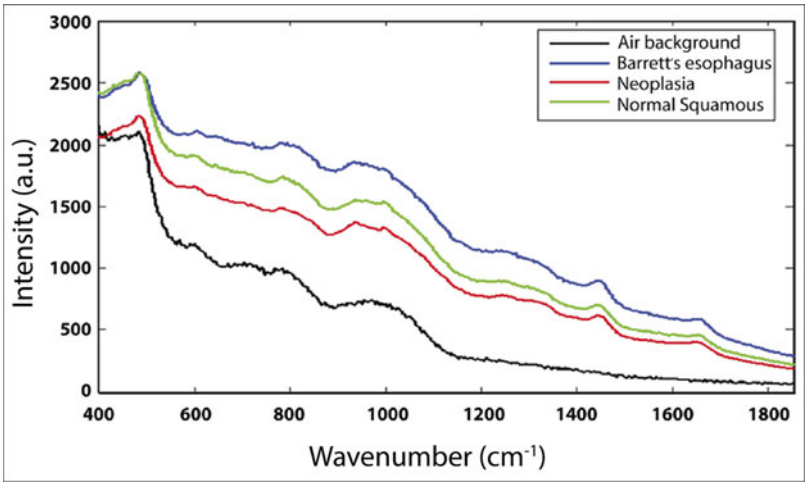

Fig. 9.8 The mean (1 s time acquired) tissue spectra for Barrett's oesophagus, adenocarcinoma, and normal squamous esophagus. Reproduced with permission form Ref. [83]

Raman Spectroscopy (RS)

Raman Spectroscopy (RS) has the benefits of utilizing tissue biochemistry changes, instead of changes in size and shape of suborganelles and cells, associated with neoplastic progression in esophageal tissue. RS measures the subtle inelastic scattering signals, obtained when incident light undergoes wavelength shifts due to transfer of energy from the incident light to the tissue, following monochromatic laser excitation. This technology has been used in the esophagus to identify neoplasia. Almond et al. [82] performed Raman spectroscopic measurements on tissue samples from 28 patients using a custom-built fiber-optic Raman probe, in conjunction with multivariate classification models, to differentiate between benign and neoplastic esophageal cancer and precancer. The Raman probe system was able to differentiate between normal squamous, Barrett's oesphagus, and neoplasia with sensitivities of (83–86 %) and specificities of (89–99 %). In another study by the same group [83, 84], using tissue from 62 patients, the authors demonstrated that the Raman probe system, in conjunction with Principal component fed linear discriminant analysis, was able to achieve a sensitivity of 86 % and a specificity of 88 % for detecting high-grade dysplasia and adenocarcinoma (Fig. 9.8).

This technology has been translated to an in vivo setting in combination with multimodal wide-field endoscopic imaging (white light reflectance (WLR) imaging, narrow-band imaging (NBI) and autofluorescence imaging (AFI) guidance (Fig. 9.9) [85, 86]. In this 25 patient study, Bergholt et al. used this multimodal system in combination with biomolecular modeling (non-negativity-constrained least-squares minimization (NNCLSM) to construct a diagnostic model giving rise

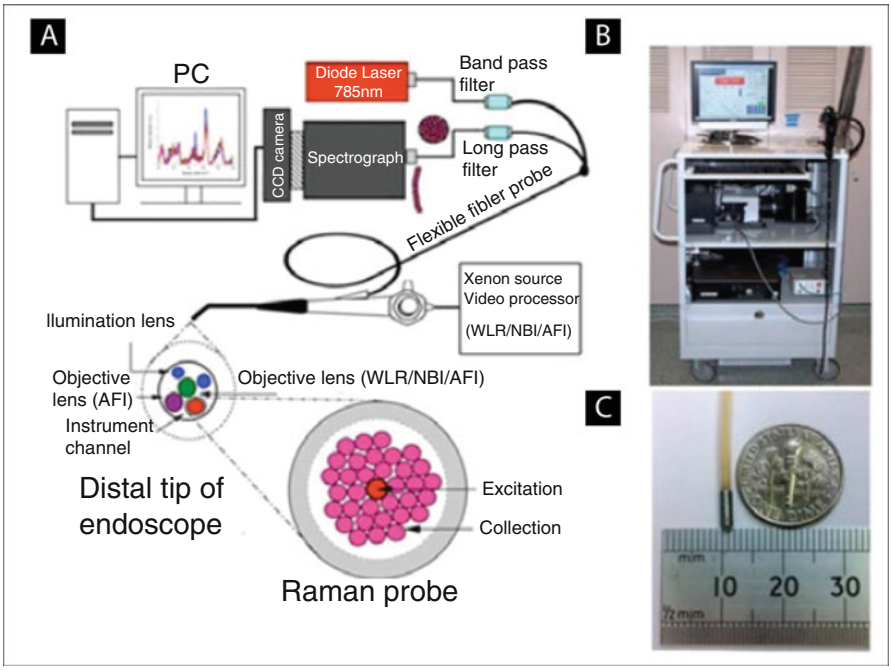

Fig. 9.9 (**a**) Schematic of the integrated Raman spectroscopy and trimodal endoscopic imaging system developed for in vivo tissue Raman measurements at endoscopy; (**b**) Photo of the Raman endoscopic system in clinic; (**c**) Photo of the fiber-optic Raman endoscopic probe. Reproduced with permission from Ref. [92]

to an accuracy of 96.0 % (i.e., sensitivity of 97.0 % and specificity of 95.2 %) for in vivo diagnosis of esophageal cancer.

Raman spectroscopy has the potential to be used for lymph node staging in real time and has been used in a number of studies to detect and classify metastatic involvement of lymph nodes in a range of cancer types [87–92]. Smith et al. demonstrated correct classification for cancerous nodes with 81 % sensitivity and 97 % specificity using Raman spectroscopy while Lloyd et al. [88] using Raman spectroscopy in combination with using principal component analysis followed by linear discriminant analysis (PCA-LDA), and by partial least squares discriminant analysis (PLS-DA) demonstrated sensitivities and specificities of 90 and 86 % were obtained using PCA-LDA, and 89 and 88 % using PLS-DA to distinguish between reactive and malignant head and neck lymph nodes. Using a novel Raman spectroscopy probe system, Horsnell et al. [89], in a study of 58 patients, were able to demonstrate sensitivities of up to 81 % and specificities of up to 97 % when differentiating between diseased and normal lymph nodes from axillary lymph nodes in breast cancer. At present, the objective of in vivo Raman analysis of lymph nodes has yet to be realized, but much research continues in this field.

Conclusion

ER offers the potential for simultaneous curative treatment and accurate staging of early esophageal cancer, and as such is the treatment of choice for such lesions. EUS is less accurate for staging T1 disease but can aid the decision of whether to use ER as first line treatment, and provide important nodal staging where ER is considered as complete local therapy for T1bsm1 disease in high-risk surgical candidates. For more advanced lesions, EUS provides the most accurate information on depth of invasion and locoregional nodal disease, while CT and PET are most useful for detecting distant metastases. Staging laparoscopy has an important role in detecting smaller foci of disease in the peritoneum and liver that may be missed on CT and PET. These different modalities should be considered complimentary, and a combined approach provides the greatest chance of accurate staging. Future developments in optical diagnosis may extend the possibilities for real-time endoscopic staging, with OCT imaging superficial neoplastic lesions and Raman probes analyzing lymph nodes for metastases.

References

1. Allum WH, Blazeby JM, Griffin SM, et al. Guidelines for the management of oesophageal and gastric cancer. Gut. 2011;60:1449–72. doi:10.1136/gut.2010.228254.
2. Rice TW, Rusch VW, Ishwaran H, et al. Cancer of the esophagus and esophagogastric junction: data-driven staging for the seventh edition of the American Joint Committee on Cancer/International Union Against Cancer Cancer Staging Manuals. Cancer. 2010;116:3763–73. doi:10.1002/cncr.25146.
3. Rice TW, Blackstone EH, Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. Ann Surg Oncol. 2010;17:1721–4. doi:10.1245/s10434-010-1024-1.
4. Raimes S. Oesophagogastric surgery. A companion to specialist surgical practice. Fourth. Saunders Elsevier; 2009.
5. Fitzgerald RC, di Pietro M, Ragunath K, et al. British Society of Gastroenterology guidelines on the diagnosis and management of Barrett's oesophagus. Gut. 2014;63:7–42. doi:10.1136/gutjnl-2013-305372.
6. Buskens CJ, Westerterp M, Lagarde SM, et al. Prediction of appropriateness of local endoscopic treatment for high-grade dysplasia and early adenocarcinoma by EUS and histopathologic features. Gastrointest Endosc. 2004;60:703–10. http://www.ncbi.nlm.nih.gov/pubmed/15557945 (accessed 13 Jun 2014).
7. Westerterp M, Koppert LB, Buskens CJ, et al. Outcome of surgical treatment for early adenocarcinoma of the esophagus or gastro-esophageal junction. Virchows Arch. 2005;446:497–504. doi:10.1007/s00428-005-1243-1.
8. Stein HJ, Feith M, Bruecher BLDM, et al. Early esophageal cancer: pattern of lymphatic spread and prognostic factors for long-term survival after surgical resection. Ann Surg. 2005;242:566–73. doi:10.1097/01.sla.0000184211.75970.85. discussion 573–5.
9. Liu L, Hofstetter WL, Rashid A, et al. Significance of the depth of tumor invasion and lymph node metastasis in superficially invasive (T1) esophageal adenocarcinoma. Am J Surg Pathol. 2005;29:1079–85. http://www.ncbi.nlm.nih.gov/pubmed/16006804 (accessed 13 Jun 2014).
10. Sepesi B, Watson TJ, Zhou D, et al. Are endoscopic therapies appropriate for superficial submucosal esophageal adenocarcinoma? An analysis of esophagectomy specimens. J Am Coll Surg. 2010;210:418–27. doi:10.1016/j.jamcollsurg.2010.01.003.

11. Alvarez Herrero L, Pouw RE, van Vilsteren FG, et al. Risk of lymph node metastasis associated with deeper invasion by early adenocarcinoma of the esophagus and cardia: study based on endoscopic resection specimens. Endoscopy. 2010;42:1030–6. doi:10.1055/s-0030-1255858.
12. Barbour AP, Jones M, Brown I, et al. Risk stratification for early esophageal adenocarcinoma: analysis of lymphatic spread and prognostic factors. Ann Surg Oncol. 2010;17:2494–502. doi:10.1245/s10434-010-1025-0.
13. Rice TW, Zuccaro G, Adelstein DJ, et al. Esophageal carcinoma: depth of tumor invasion is predictive of regional lymph node status. Ann Thorac Surg. 1998;65:787–92. doi:10.1016/S0003-4975(97)01387-8.
14. Pech O, May A, Manner H, et al. Long-term efficacy and safety of endoscopic resection for patients with mucosal adenocarcinoma of the esophagus. Gastroenterology. 2014;146:652–60. e1. doi:10.1053/j.gastro.2013.11.006.
15. Prasad GA, Wu TT, Wigle DA, et al. Endoscopic and surgical treatment of mucosal (T1a) esophageal adenocarcinoma in Barrett's esophagus. Gastroenterology. 2009;137:815–23. doi:10.1053/j.gastro.2009.05.059.
16. Estrella JS, Hofstetter WL, Correa AM, et al. Duplicated muscularis mucosae invasion has similar risk of lymph node metastasis and recurrence-free survival as intramucosal esophageal adenocarcinoma. Am J Surg Pathol. 2011;35:1045–53. doi:10.1097/PAS.0b013e318219ccef.
17. Conio M, Repici A, Cestari R, et al. Endoscopic mucosal resection for high-grade dysplasia and intramucosal carcinoma in Barrett's esophagus: an Italian experience. World J Gastroenterol. 2005;11:6650–5.
18. Seewald S, Akaraviputh T, Seitz U, et al. Circumferential EMR and complete removal of Barrett's epithelium: a new approach to management of Barrett's esophagus containing high-grade intraepithelial neoplasia and intramucosal carcinoma. Gastrointest Endosc. 2003;57:854–9. doi:10.1016/S0016-5107(03)70020-0.
19. Ahmad NA, Kochman ML, Long WB, et al. Efficacy, safety, and clinical outcomes of endoscopic mucosal resection: a study of 101 cases. Gastrointest Endosc. 2002;55:390–6. doi:10.1067/mge.2002.121881.
20. Mino-Kenudson M, Brugge WR, Puricelli WP, et al. Management of superficial Barrett's epithelium-related neoplasms by endoscopic mucosal resection: clinicopathologic analysis of 27 cases. Am J Surg Pathol. 2005;29:680–6. doi:10.1097/01.pas.0000154129.87219.fa.
21. Peters FP, Brakenhoff KPM, Curvers WL, et al. Histologic evaluation of resection specimens obtained at 293 endoscopic resections in Barrett's esophagus. Gastrointest Endosc. 2008;67:604–9. doi:10.1016/j.gie.2007.08.039.
22. Pouw RE, Seewald S, Gondrie JJ, et al. Stepwise radical endoscopic resection for eradication of Barrett's oesophagus with early neoplasia in a cohort of 169 patients. Gut. 2010;59:1169–77. doi:10.1136/gut.2010.210229.
23. Konda VJA, Ross AS, Ferguson MK, et al. Is the risk of concomitant invasive esophageal cancer in high-grade dysplasia in Barrett's esophagus overestimated? Clin Gastroenterol Hepatol. 2008;6:159–64. doi:10.1016/j.cgh.2007.09.013.
24. Khanna LG, Gress FG. Preoperative evaluation of oesophageal adenocarcinoma. Best Pract Res Clin Gastroenterol. 2015;29:179–91. doi:10.1016/j.bpg.2014.12.005.
25. Kelly S, Harris KM, Berry E, et al. A systematic review of the staging performance of endoscopic ultrasound in gastro-oesophageal carcinoma. Gut. 2001;49:534–9. doi:10.1136/gut.49.4.534.
26. Young PE, Gentry AB, Acosta RD, et al. Endoscopic ultrasound does not accurately stage early adenocarcinoma or high-grade dysplasia of the esophagus. Clin Gastroenterol Hepatol. 2010;8:1037–41. doi:10.1016/j.cgh.2010.08.020.
27. Larghi A, Lightdale CJ, Memeo L, et al. EUS followed by EMR for staging of high-grade dysplasia and early cancer in Barrett's esophagus. Gastrointest Endosc. 2005;62:16–23. doi:10.1016/S0016-5107(05)00319-6.
28. Pech O, Günter E, Dusemund F, et al. Accuracy of endoscopic ultrasound in preoperative staging of esophageal cancer: results from a referral center for early esophageal cancer. Endoscopy. 2010;42:456–61. doi:10.1055/s-0029-1244022.

29. Pouw RE, Heldoorn N, Herrero LA, et al. Do we still need EUS in the workup of patients with early esophageal neoplasia? A retrospective analysis of 131 cases. Gastrointest Endosc. 2011;73:662–8. doi:10.1016/j.gie.2010.10.046.
30. Pech O, Günter E, Dusemund F, et al. Value of high-frequency miniprobes and conventional radial endoscopic ultrasound in the staging of early Barrett's carcinoma. Endoscopy. 2010;42:98–103. doi:10.1055/s-0029-1243839.
31. Catalano MF, Sivak MV, Rice T, et al. Endosonographic features predictive of lymph node metastasis. Gastrointest Endosc. 1994;40:442–6. doi:10.1016/S0016-5107(94)70206-3.
32. Bhutani MS, Hawes RH, Hoffman BJ. A comparison of the accuracy of echo features during endoscopic ultrasound (EUS) and EUS-guided fine-needle aspiration for diagnosis of malignant lymph node invasion. Gastrointest Endosc. 1997;45:474–9. doi:10.1016/S0016-5107(97)70176-7.
33. Rösch T. Endosonographic staging of esophageal cancer: a review of literature results. Gastrointest Endosc Clin N Am. 1995;5:537–47.
34. Räsänen JV, Sihvo EIT, Knuuti MJ, et al. Prospective analysis of accuracy of positron emission tomography, computed tomography, and endoscopic ultrasonography in staging of adenocarcinoma of the esophagus and the esophagogastric junction. Ann Surg Oncol. 2003;10:954–60. doi:10.1245/ASO.2003.12.002.
35. Vazquez-Sequeiros E, Norton ID, Clain JE, et al. Impact of EUS-guided fine-needle aspiration on lymph node staging in patients with esophageal carcinoma. Gastrointest Endosc. 2001;53:751–7. doi:10.1067/mge.2001.112741.
36. Wiersema MJ, Vilmann P, Giovannini M, et al. Endosonography-guided fine-needle aspiration biopsy: diagnostic accuracy and complication assessment. Gastroenterology. 1997;112:1087–95.
37. Sandha GS, Severin D, Postema E, et al. Is positron emission tomography useful in locoregional staging of esophageal cancer? Results of a multidisciplinary initiative comparing CT, positron emission tomography, and EUS. Gastrointest Endosc. 2008;67:402–9. doi:10.1016/j.gie.2007.09.006.
38. Keswani RN, Early DS, Edmundowicz SA, et al. Routine positron emission tomography does not alter nodal staging in patients undergoing EUS-guided FNA for esophageal cancer. Gastrointest Endosc. 2009;69:1210–7. doi:10.1016/j.gie.2008.08.016.
39. Pech O, May A, Günter E, et al. The impact of endoscopic ultrasound and computed tomography on the TNM staging of early cancer in Barrett's esophagus. Am J Gastroenterol. 2006;101:2223–9. doi:10.1111/j.1572-0241.2006.00718.x.
40. Bergeron EJ, Lin J, Chang AC, et al. Endoscopic ultrasound is inadequate to determine which T1/T2 esophageal tumors are candidates for endoluminal therapies. J Thorac Cardiovasc Surg. 2014;147:765–73. doi:10.1016/j.jtcvs.2013.10.003.
41. Meister T, Domagk D, Heinzow HS, et al. Miniprobe endoscopic ultrasound accurately stages esophageal cancer and guides therapeutic decisions in the era of neoadjuvant therapy: results of a multicenter cohort analysis. Surg Endosc. 2013;27:2813–9. doi:10.1007/s00464-013-2817-7.
42. Bulsiewicz WJ, Dellon ES, Rogers AJ, et al. The impact of endoscopic ultrasound findings on clinical decision making in Barrett's esophagus with high-grade dysplasia or early esophageal adenocarcinoma. Dis Esophagus. 2014;27:409–17. doi:10.1111/j.1442-2050.2012.01408.x.
43. Godoy MCB, Bruzzi JF, Viswanathan C, et al. Multimodality imaging evaluation of esophageal cancer: staging, therapy assessment, and complications. Abdom Imaging. 2013;38:974–93. doi:10.1007/s00261-013-9986-7.
44. Rice TW. Clinical staging of esophageal carcinoma. CT, EUS, and PET. Chest Surg Clin N Am. 2000;10:471–85.
45. Van Vliet EPM, Heijenbrok-Kal MH, Hunink MGM, et al. Staging investigations for oesophageal cancer: a meta-analysis. Br J Cancer. 2008;98:547–57. doi:10.1038/sj.bjc.6604200.
46. Quint LE, Hepburn LM, Francis IR, et al. Incidence and distribution of distant metastases from newly diagnosed esophageal carcinoma. Cancer. 1995;76:1120–5. doi:10.1002/1097-0142(19951001)76:7<1120::AID-CNCR2820760704>3.0.CO;2-W.
47. Lowe VJ, Booya F, Fletcher JG, et al. Comparison of positron emission tomography, computed tomography, and endoscopic ultrasound in the initial staging of patients with esophageal cancer. Mol Imaging Biol. 2005;7:422–30. doi:10.1007/s11307-005-0017-0.

48. Lin J, Kligerman S, Goel R, et al. State-of-the-art molecular imaging in esophageal cancer management : implications for diagnosis, prognosis, and treatment. J Gastrointest Oncol. 2015;6:3–19. doi:10.3978/j.issn.2078-6891.2014.062.
49. Erasmus JJ, Munden RF. The role of integrated computed tomography positron-emission tomography in esophageal cancer: staging and assessment of therapeutic response. Semin Radiat Oncol. 2007;17:29–37. doi:10.1016/j.semradonc.2006.09.005.
50. Bruzzi JF, Munden RF, Truong MT, et al. PET/CT of esophageal cancer: its role in clinical management. Radiographics. 2007;27:1635–52. doi:10.1148/rg.276065742.
51. Cuellar SLB, Carter BW, Macapinlac HA, et al. Clinical staging of patients with early esophageal adenocarcinoma. J Thorac Oncol. 2014;9:1202–6. doi:10.1097/JTO.0000000000000222.
52. Kato H, Miyazaki T, Nakajima M, et al. The incremental effect of positron emission tomography on diagnostic accuracy in the initial staging of esophageal carcinoma. Cancer. 2005;103:148–56. doi:10.1002/cncr.20724.
53. Stahl A, Stollfuss J, Ott K, et al. FDG PET and CT in locally advanced adenocarcinomas of the distal oesophagus: clinical relevance of a discordant PET finding. Nuklear Medizin. 2005;44:249–55. doi:05060249 [pii].
54. Al-Taan OS, Eltweri A, Sharpe D, et al. Prognostic value of baseline FDG uptake on PET-CT in esophageal carcinoma. World J Gastrointest Oncol. 2014;6:139–44. doi:10.4251/wjgo.v6.i5.139.
55. Vallböhmer D, Hölscher AH, Dietlein M, Bollschweiler E, Baldus SE, Mönig SP, Metzger R, Schicha HSM. [18F]-Fluorodeoxyglucose-positron emission tomography for the assessment of histopathologic response and prognosis after completion of neoadjuvant chemoradiation in esophageal cancer. Ann Surg. 2009;250:888–94.
56. Smith A, Finch MD, John TG, et al. Role of laparoscopic ultrasonography in the management of patients with oesophagogastric cancer. Br J Surg. 1999;86:1083–7. doi:10.1046/j.1365-2168.1999.01190.x.
57. Rau B, Hünerbein M. Diagnostic laparoscopy: indications and benefits. Langenbeck's Arch Surg. 2005;390:187–96. doi:10.1007/s00423-004-0483-x.
58. Nath J, Moorthy K, Taniere P, et al. Peritoneal lavage cytology in patients with oesophagogastric adenocarcinoma. Br J Surg. 2008;95:721–6. doi:10.1002/bjs.6107.
59. De Graaf GW, Ayantunde AA, Parsons SL, et al. The role of staging laparoscopy in oesophagogastric cancers. Eur J Surg Oncol. 2007;33:988–92. doi:10.1016/j.ejso.2007.01.007.
60. Compton CC, Byrd DR, Garcia-Aguilar J, et al., editors. AJCC Cancer Staging Atlas. New York, NY: Springer; 2012. doi: 10.1007/978-1-4614-2080-4.
61. Li-Qing Y. New endoscopic diagnosis and treatment options for early esophageal cancer. J Gastrointest Dig Syst. 2012.
62. Pierce MC, Javier DJ, Richards-Kortum R. Optical contrast agents and imaging systems for detection and diagnosis of cancer. Int J Cancer. 2008;123(9):1979–90.
63. Testoni PA, Mangiavillano B. Optical coherence tomography in detection of dysplasia and cancer of the gastrointestinal tract and bilio-pancreatic ductal system. World J Gastroenterol. 2008;14(42):6444.
64. Tsai T-H, Fujimoto JG, Mashimo H. Endoscopic optical coherence tomography for clinical gastroenterology. Diagnostics. 2014;4(2):57–93.
65. Isenberg G, et al. Accuracy of endoscopic optical coherence tomography in the detection of dysplasia in Barrett's esophagus: a prospective, double-blinded study. Gastrointest Endosc. 2005;62(6):825–31.
66. Evans JA, et al. Optical coherence tomography to identify intramucosal carcinoma and high-grade dysplasia in Barrett's esophagus. Clin Gastroenterol Hepatol. 2006;4(1):38–43.
67. Qi X, et al. Image analysis for classification of dysplasia in Barrett's esophagus using endoscopic optical coherence tomography. Biomed Optic Express. 2010;1(3):825–47.
68. Chen Y, et al. Ultrahigh resolution optical coherence tomography of Barrett's esophagus: preliminary descriptive clinical study correlating images with histology. Endoscopy. 2007;39(7):599–605.
69. Jäckle S, et al. In vivo endoscopic optical coherence tomography of esophagitis, Barrett's esophagus, and adenocarcinoma of the esophagus. Endoscopy. 2000;32(10):750–5.

70. Li XD, et al. Optical coherence tomography: advanced technology for the endoscopic imaging of Barrett's esophagus. Endoscopy. 2000;32(12):921–30.
71. Lightdale CJ. Advanced imaging for GI endoscopy. Gastrointest Endosc Clin N Am. 2013;23(3):xiii–xiv.
72. Robles LY, Satish S, Fisichella PM. Emerging enhanced imaging technologies of the esophagus: spectroscopy, confocal laser endomicroscopy, and optical coherence tomography. J Surg Res. 2015;195(2):502–14.
73. Singh R, Yeap SP. Endoscopic imaging in Barrett's esophagus. Expert Rev Gastroenterol Hepatol. 2014:1–11
74. Wang KK. Detection and staging of esophageal cancers. Curr Opin Gastroenterol. 2004;20(4):381.
75. Hatta W, et al. A prospective comparative study of optical coherence tomography and EUS for tumor staging of superficial esophageal squamous cell carcinoma. Gastrointest Endosc. 2012;76(3):548–55.
76. Hatta W, et al. Optical coherence tomography for the staging of tumor infiltration in superficial esophageal squamous cell carcinoma. Gastrointest Endosc. 2010;71(6):899–906.
77. McLaughlin RA, et al. Imaging of human lymph nodes using optical coherence tomography: potential for staging cancer. Cancer Res. 2010;70(7):2579–84.
78. McLaughlin RA, et al. Imaging of breast cancer with optical coherence tomography needle probes: feasibility and initial results. Selected topics in Quantum electronics. IEEE J. 2012;18(3):1184–91.
79. Standish BA, et al. Interstitial Doppler optical coherence tomography as a local tumor necrosis predictor in photodynamic therapy of prostatic carcinoma: an in vivo study. Cancer Res. 2008;68(23):9987–95.
80. Iftimia NV, et al. Spectral-domain low coherence interferometry/optical coherence tomography system for fine needle breast biopsy guidance. Rev Sci Instrum. 2009;80(2):024302.
81. Kirtane TS, Wagh MS. Endoscopic optical coherence tomography (OCT): advances in gastrointestinal imaging. Gastroenterol Res Pract. 2014;2014:376367.
82. Almond LM, et al. Assessment of a custom-built Raman spectroscopic probe for diagnosis of early oesophageal neoplasia. J Biomed Opt. 2012;17(8):0814211–6.
83. Almond LM, et al. Endoscopic Raman spectroscopy enables objective diagnosis of dysplasia in Barrett's esophagus. Gastrointest Endosc. 2014;79(1):37–45.
84. Almond LM, et al. Real-time disease detection using spectroscopic diagnosis. Biomed Spectros Imag. 2014;3(3):197–202.
85. Bergholt MS, et al. In vivo diagnosis of esophageal cancer using image-guided Raman endoscopy and biomolecular modeling. Technol Cancer Res Treat. 2011;10(2):103–12.
86. Bergholt MS et al. Raman endoscopy for objective diagnosis of early cancer in the gastrointestinal system. J Gastroint Dig Syst. 2013;S1:008.
87. Isabelle M, et al. Lymph node pathology using optical spectroscopy in cancer diagnostics. J Spectros. 2008;22(2–3):97–104.
88. Lloyd GR, et al. Discrimination between benign, primary and secondary malignancies in lymph nodes from the head and neck utilising Raman spectroscopy and multivariate analysis. Analyst. 2013;138(14):3900–8.
89. Horsnell JD, et al. Raman spectroscopy—a potential new method for the intra-operative assessment of axillary lymph nodes. Surgeon. 2012;10(3):123–7.
90. Horsnell J, et al. Intra-operative Sentinel Lymph Node Assessment-How many patients will avoid a second operation? Eur J Surg Oncol. 2012;38(5):459–60.
91. Smith J, et al. Raman spectral mapping in the assessment of axillary lymph nodes in breast cancer. Technol Cancer Res Treat. 2003;2(4):327–31.
92. Day JCC, Stone N. A subcutaneous Raman needle probe. Appl Spectrosc. 2013;67(3):349–54.

Chapter 10
Transcommitment: Paving the Way to Barrett's Metaplasia

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Barrett's esophagus is the metaplastic change of the distal esophageal epithelium from stratified squamous to columnar that can arise as a complication of gastroesophageal reflux disease (GERD) [1]. Barrett's esophagus is considered by many as an adaptive response to chronic exposure to gastric acid and intestinal bile salts since a columnar-lined esophagus (CLE) should be more protective against these insults. The metaplastic esophageal columnar epithelium can be one of three types: specialized intestinal metaplasia, characterized by (intestinal mucin) MUC2-expressing goblet cells and other intestinal cell types; cardia-type epithelium, characterized by mucus containing cells; and gastric fundic-type epithelium, characterized by mucus secreting, parietal, and chief cells [2]. While all three types of CLE are classified as Barrett's metaplasia outside of the United States, the American Gastroenterological Association (AGA) more strictly defines Barrett's esophagus as "the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus" [2]. Since only specialized intestinal metaplasia is clearly at increased risk of transforming into esophageal adenocarcinoma, the more prevalent form of esophageal cancer in Western

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countries, the presence of goblet cells is required for the histopathologic definition of Barrett's esophagus in the United States [2].

Metaplasia is the replacement of one fully differentiated tissue by another [3]. In Barrett's esophagus, the columnar epithelium that replaces the squamous epithelium originates either from a native esophageal cell or from a cell external to the esophagus that relocates to the esophagus and undergoes molecular reprogramming (i.e., transdifferentiation or transcommitment). Wherever the source of the Barrett's esophagus cell or tissue of origin, the original cell is unlikely intestinal and therefore must undergo molecular reprogramming to convert into epithelium classified as specialized intestinal metaplasia. Furthermore, the columnar cell would need to possess or acquire the ability to form three-dimensional (3D) structures such as the glands observed in Barrett's esophagus.

There are four general possibilities for the source of the Barrett's esophagus cell or tissue of origin (Table 10.1). First, a native esophageal differentiated squamous cell could give rise to an intestinal columnar cell through irreversible direct phenotypic conversion, also known as transdifferentiation. Second, a native esophageal progenitor cell, which could be located within the squamous epithelium or in a submucosal gland or its duct, differentiates to become an intestinal columnar cell (esophageal progenitor cell transcommitment). Third, an external circulating bone marrow-derived stem cell migrates to the esophagus and undergoes intestinal columnar epithelial differentiation (circulating stem cell transcommitment). Fourth, an external columnar progenitor cell from the squamocolumnar junction (SCJ) or gastric cardia proximally shifts to fill a void left by damaged stratified squamous epithelium and then undergoes intestinal differentiation (columnar progenitor cell transcommitment).

Identifying the cell or tissue origin of Barrett's esophagus could lead to development of effective treatment or prevention strategies for this condition. Prevention is especially relevant in current clinical practice where Barrett's esophagus is often treated with radiofrequency ablation (RFA) and frequently recurs postablation [4]. Understanding the process of how Barrett's esophagus forms could also provide new insights into normal tissue development and differentiation, wound healing, and stem cell biology. Data supporting various possible sources of the Barrett's esophagus cell or tissue of origin have arisen from patient biopsies or human esophagectomy specimens; in vivo animal models (i.e., surgically induced reflux esophagitis or genetically engineered mice); cell culture experiments utilizing human esophageal squamous and Barrett's epithelial cell lines; and novel experimental systems such as organ explant culture, 3D organotypic culture, or in vivo transplant culture.

Transdifferentiation

Support for transdifferentiation is derived from the description of multilayered epithelium (MLE), demonstrating the existence of biphenotypic squamous and columnar cells in adult human patients and experimental animals. By scanning

Table 10.1 Summary evidence transdifferentiation and transcommitment

Type of molecular reprogramming/cell source	Reasons supporting	Reasons opposing
Transdifferentiation		
Esophageal differentiated squamous cell	Multilayered epithelium (MLE)	No full phenotypic conversion of cultured cell in vitro Not likely to sustain the tissue
Transcommitment		
Esophageal progenitor cell	Biphenotypic cell in embryonic esophagus	
Squamous	Hierarchy of progenitor/ differentiated cells	No direct evidence
Submucosal gland or duct	Gland ducts contiguous with squamous, multilayered, metaplastic Barrett's, and neosquamous epithelium (with shared DNA and mitochondrial DNA mutations)	Rodents don't possess submucosal glands but develop Barrett's esophagus following surgical induction of reflux esophagitis
Circulating bone marrow-derived stem cell	Transplant donor cells contribute to esophageal squamous and metaplastic Barrett's epithelium and adenosquamous cancer	Donor cells only *partially* contribute to glands
Proximally shifting columnar progenitor cell		Barrett's esophagus occurs in patients following esophagectomy in which the SCJ and gastric cardia are removed
Squamo-columnar junction (SCJ)	Residual embryonic cells proximally shift following injury to adjacent squamous epithelium in mice	
Gastric cardia	"Creeping substitution" occurs in dog models of reflux esophagitis Metaplastic glands observed in mice with IL-1β or Bmp4 overexpression or Smad3 deletion	

electron microscopy (SEM), a distinctive superficial "transition zone cell" was identified in mucosal biopsies from patients with Barrett's esophagus where squamous and Barrett's epithelium were apposed [5]. Squamous epithelium was characterized by SEM as having prominent intercellular ridges and distinct microridges while every Barrett's esophagus cell exhibited microvilli and had neither intercellular ridges nor microridges. In contrast to both squamous and Barrett's epithelium, transition zone cells displayed both intercellular ridges and short, stubby microvilli with some transition zone cells also demonstrating bulging mucus. Thus, these distinctive transition zone cells possessed SEM features of both squamous and columnar epithelium. In addition, SEM could

further differentiate gastric surface-like cells from intestinal absorptive-like cells from goblet cells, which contained a central mound of protruding mucus, by surface topology. The investigators next examined biopsies taken from the normal gastroesophageal junction (GEJ) from several patients and did not find cells resembling transition zone cells, suggesting that transition zone cells were unique to Barrett's esophagus in the gastrointestinal (GI) tract. Later investigations found these distinctive transition zone cells overlying squamous epithelial cells in areas of squamo-Barrett's transition [6]. Now termed "multilayered epithelium," cytokeratin (CK) expression analysis by immunohistochemistry (IHC) demonstrated that individual basal cells found in MLE simultaneously expressed both squamous CK4 and columnar CK19 [7]. Mucin expression profiling of both basal and superficial cells showed that MLE more closely resembled Barrett's epithelium and esophageal submucosal gland duct epithelium than gastric cardia epithelium, arguing against proximal shifting of gastric cardia cells [8]. The observation of MLE in association with Barrett's esophagus in rats that have undergone esophagogastroduodenal anastomosis to surgically induce bile reflux suggested that MLE could be a precursor lesion to Barrett's esophagus [9, 10]. Ultrastructural analysis of mouse esophageal basal epithelial cells in which the intestinal transcription factor Cdx2 is overexpressed using the CK14 promoter revealed that the cells acquired some characteristics of MLE with both squamous and secretory features [11].

A common objection to transdifferentiation as a mechanism of Barrett's metaplasia is that direct phenotypic conversion of a squamous esophageal cell into an intestinal goblet cell has not yet been achieved in vitro. Overexpression of various transcription factors in differentiated human esophageal squamous cell lines such as HET-1A (SV-40 immortalized) [12, 13], EPC2 (hTERT immortalized, proximal esophagus) [14, 15], NES-B3T, and NES-B10T (hTERT immortalized, distal esophagus) [16, 17] has led to expression of columnar, intestinal, or mucin associated genes that are found in Barrett's esophagus but have not led to full phenotypic conversion of a squamous cell into a specialized intestinal metaplastic cell. It is likely that a less differentiated cell that still maintains multipotent potential (i.e., a progenitor or stem cell) is required to form an intestinal goblet cell under these conditions.

Native Esophageal Squamous Progenitor Cells

Evidence for transcommitment of esophageal epithelial progenitor cells comes from observations made during esophageal embryogenesis. The entire GI tract, including the esophagus, embryologically develops from a columnar epithelium-lined gut tube that then regionalizes and undergoes organ-specific differentiation. In humans, the epithelium lining the esophagus begins as a pseudostratified columnar epithelium, seen as early as 7 weeks gestational age in autopsy specimens [18]. By 8–10 weeks gestational age, the esophageal epithelium has become ciliated and

simple columnar. Early in the 5th month of gestation, the epithelium changes from columnar to squamous near the mid-esophagus [19]. The entire esophageal epithelium then bidirectionally transitions into stratified squamous from this starting point, concluding at both the proximal and distal ends of the esophagus. IHC staining revealed expression of squamous CK5 and CK13 in the 14-week-old embryo; however, columnar CK20 was still observed in esophageal epithelium in the 17-week-old embryo [18]. Based on these observations, either a mixed phenotype of squamous and columnar epithelium may line the esophagus prior to its final differentiation into stratified squamous beginning at 5 months of gestation or a subpopulation of epithelial cells may express both columnar and squamous CK's simultaneously.

This phenotypic switch was subsequently more carefully studied in mouse embryos. In mice, normal esophageal epithelial development occurs between E (embryonic day) 11.5, when the esophagus separates from the trachea, and 1 month after birth when the esophageal epithelium fully keratinizes. The timing of this change varies based on the mouse strain [20–22]. It was initially thought that the mechanism causing epithelial phenotype switching from columnar to stratified squamous was luminal sloughing of columnar cells followed by replacement by underlying squamous cells. However, an elegant study using outbred CD1 mice demonstrated otherwise [22]. Investigators from the Tosh lab found that during normal esophageal embryogenesis some epithelial cells simultaneously expressed columnar and squamous CKs. This was demonstrated using columnar CK8 immunostaining in conjunction with a squamous CK14-nuclear green fluorescent protein (GFP) reporter or with CK8 and CK14 coimmunostaining. The acquisition of expression of a squamous CK by the CK8 positive columnar cells occurred in the absence of cell division and ended with CK8 being silenced through de novo promoter methylation.

Multiple studies have characterized adult mouse esophageal epithelial progenitor cells and identified several cell surface markers and populations. Epperly and colleagues harvested esophageal epithelium from male GFP positive C57BL/6 mice and isolated esophageal epithelial progenitor cells using serial preplating or sorting for side population cells after staining with Hoechst dye [23]. Side population cells are characterized by their ability to exclude the DNA dye Hoechst 33342, and this technique has been used successfully to identify stem cells [24]. Identified esophageal epithelial progenitor cells had high expression of the cell surface markers Sca-1 and Thy-1, homed to the esophagus when injected into the tail vein of female GFP negative mice that had been irradiated to induce esophagitis, and gave rise to colonies of cells within the esophagus. These GFP positive cells also could be serially passaged in later generations of female GFP negative mice. Though it was likely that the serially passaged GFP positive cells were epithelial, no costaining experiments were performed to confirm this.

Kalabis and colleagues identified a population of label retaining cells (LRC), using BrdU or tritiated thymidine incorporation, comprising approximately 1 % of all CK14 positive basal epithelial cells in the mouse esophagus [25]. Since either type of DNA label is diluted by successive cell divisions, this technique can be used

to identify the slowest cycling cells which many consider to be quiescent progenitor or stem cells. These LRC were proven to be epithelial by staining with pan-CK and could be sorted among side population cells following staining with Hoechst dye. Side population cells contained a higher percentage of CD34 positive cells compared to the total esophageal epithelial cell population (34 % versus 0.4 %), formed colonies, and made a fully differentiated epithelium with keratinization in 3D organotypic culture. The 3D organotypic cultures made with side population cells also had more robust immunostaining for CK4 and CK13 which are normally expressed by suprabasal layers of squamous epithelium, and cells from these cultures could produce second-generation 3D organotypic cultures with a similar mature CK differentiation pattern. Finally, in mice in which the esophageal epithelium was mechanically wounded GFP positive, CD34 positive cells were shown to participate in repair within 48 h while GFP positive, CD34 negative cells did not. Based on these results, it was concluded that CD34 positive LRC represented esophageal epithelial progenitor cells.

Croagh and colleagues divided esophageal basal epithelial cells into four groups based on their expression of α6 integrin and CD71 [26]. Cells that were $\alpha6^{bright}CD71^{dim}$, $\alpha6^{bright}CD71^{bright}$, or $\alpha6^{dim}$ had similar clonogenic potential compared to cells that were CD71 very bright ($CD71^{+++}$) which had a much lower clonogenic potential. In an in vivo transplant culture system in which mouse esophageal epithelial cells were injected into a denuded rat trachea which was then placed subcutaneously into NOD/SCID mice, $\alpha6^{bright}CD71^{dim}$ and $\alpha6^{bright}CD71^{bright}$ cells generated a differentiated stratified squamous epithelium while $\alpha6^{dim}$ cells did not. Finally, $\alpha6^{bright}CD71^{dim}$ cells were shown to have the highest number of LRC for the longest period of time, consistent with being the least differentiated progenitor cells. DeWard and colleagues also identified α6 integrin as a marker of cells that have stem cell-like features [27]. Single cells isolated from mouse esophageal epithelium gave rise to organoids in growth media supplemented with exogenous stem cell factors. This ability to form organoids in culture is a property retained by stem cells [28]. Further analysis found that this organoid-forming ability was dependent on positive Sox2 expression and limited to cells located in the basal layer. Sox2 positive basal cells were further sorted based on expression of α6 integrin, β4 integrin, and CD73. α6/β4 integrin high, CD73 positive cells possessed the highest organoid forming activity. Treatment with the differentiating agent all-trans retinoic acid (ATRA) led to a decrease in α6/β4 integrin high, CD73 positive or α6/β4 integrin high, CD73 negative cells and an increase in α6/β4 integrin low cells. These studies support a hierarchy of cells within the basal epithelial cell layer, with α6/β4 integrin high, CD73 positive cells being the least differentiated.

Further work on identifying progenitor cells in the mouse esophagus was performed in the Jones lab [29]. They identified 0.4 % of esophageal basal epithelial cells as LRC, but these LRC did not stain for CK14 or CD34 as Kalabis and colleagues had reported. Instead these LRC stained for CD45, consistent with a hematopoietic origin. Using a tamoxifen-inducible Ahcre mouse line [30] to activate a yellow fluorescent protein (YFP) reporter, these investigators tracked individual cell clones in the mouse esophagus over 1 year. Unexpectedly, the number of YFP

positive clones decreased while the size of each clone increased linearly over time. Based on these findings, Doupé and colleagues hypothesized that all esophageal progenitor cells were functionally equivalent, stochastically dividing to equally give rise to proliferating and differentiating daughter cells, and that the esophageal epithelium did not have a slow cycling stem cell population. Mice were then treated with ATRA at a dose selected to induce hyperproliferation and lineage tracing was performed. ATRA doubled both basal cell proliferation and differentiation with no observed difference in the proportion of symmetric (proliferating daughter cells) versus asymmetric (progenitor cells) cell division, thus establishing a new homeostatic state. The mouse esophageal epithelium was next injured by performing a microendoscopic biopsy to investigate the role of esophageal progenitors in wound repair. Through the use of two separate reporter mouse strains and EdU incorporation experiments, these investigators demonstrated that injury caused esophageal progenitors to favor proliferation over differentiation for a period of 5 days after which they returned to equal states of proliferation and differentiation.

Unlike mouse esophageal epithelium which is typically divided into basal and suprabasal compartments, the human esophageal epithelium is divided into two basal cell regions: one overlying the stromal papillae and the other overlying interpapillary regions [31] (Fig. 10.1). Seery and Watt described cells in the interpapillary basal cell regions as undergoing asymmetric division, while proliferating cells overlying papillae underwent symmetric division. Proliferating Ki-67 positive cells were found to be four times more common in cells overlying papillae, which had higher levels of β1 integrin. In contrast, studies from the Fitzgerald lab demonstrated that in human esophageal epithelium the most

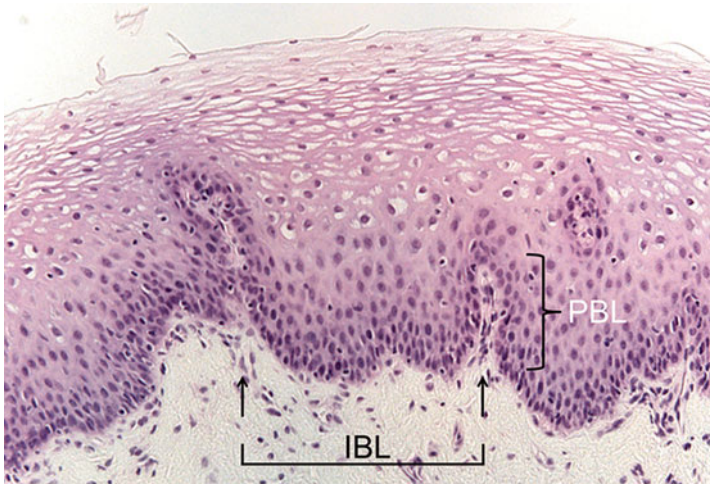

Fig. 10.1 Human esophagus (20×). Stromal papillae are indicated by the *arrows*. Basal cells found between two papillae are within the interpapillary basal cell layer or region (IBL). Basal cells overlying papillae are within the papillary basal cell layer or region (PBL) which is indicated by the curly brace

proliferative basal cells are found within the interpapillary region with no mitoses seen in the region overlying the tips of the papillae [32]. Cells at the tip of the papillae were quiescent and costained for CD34 and β1 integrin. Barbera and colleagues then sorted human esophageal cells into four groups using a CD34 antibody and an antibody against EpCAM, a marker for cells in the suprabasal layer. They found no difference in clonogenic capacity between the four groups and concluded that esophageal epithelial progenitor cells were widespread, not restricted to either basal cell region, and included cells that were committed to epithelial differentiation. In another study, Pan and colleagues injected four patients with adenocarcinoma who were scheduled to undergo esophagectomy with IdU to label proliferative cells and collected esophageal squamous tissue at the time of surgical resection 7, 11, 29, and 67 days postinjection [33]. Using IHC for Ki-67, they found that proliferating cells were located within the basal epithelium of both interpapillary and papillary regions and should have incorporated IdU during the infusion. After 7 days, IdU positive squamous cells were observed within the basal layer as well as in differentiating cells transiting toward the lumen. By 29 and 67 days postinjection, LRC were rare and limited to basal epithelium. In agreement with studies by Barbera and colleagues [32], LRC were concentrated in the basal cell region overlying papillae and costained with pan-CK confirming an epithelial phenotype. In addition, these LRC were located in close proximity to Ki-67 positive proliferating cells suggesting that perhaps these LRC gave rise to proliferative Ki-67 positive cells.

A conclusion drawn from these studies is that esophageal squamous cells likely have a hierarchy in terms of differentiation status but may not contain true stem cells. If squamous cells in the basal layer of the esophagus are the precursor cell pool then these cells could undergo transcommitment to give rise to Barrett's esophagus. If, on the other hand, cells throughout the stratified cell layers as well as cells in the basal layer equally function as progenitor cells, then transdifferentiation would be functionally synonymous with transcommitment. Direct evidence of a squamous progenitor cell undergoing a full phenotypic conversion into a specialized intestinal metaplastic cell remains lacking. Lineage tracing experiments using reporter mice may resolve this in the future.

Native Esophageal Submucosal Gland or Duct Progenitor Cells

Submucosal glands are found in the human esophagus and are distributed throughout its entirety [34, 35]. A single submucosal gland consists of acini lined by simple columnar cells surrounded by myoepithelial cells and a single duct lined by cuboidal cells that transition into squamous cells as the duct reaches the esophageal lumen [35]. Acinar secretions include acidic and neutral mucins, bicarbonate, epidermal growth factor, and prostaglandins. Submucosal gland ducts have been observed in direct continuity with overlying squamous epithelium, MLE, Barrett's epithelium, and neosquamous islands found within areas of Barrett's esophagus [8, 34, 36] supporting the hypothesis that these glands harbor epithelial progenitor cells. The existence of

progenitor cells within submucosal glands or their ducts was further supported by clonal studies tracking DNA mutations [37]. Using laser capture microdissection and DNA sequencing, Leedham and colleagues located a squamous island arising from a submucosal gland duct. While a surrounding area of Barrett's metaplasia was P53 mutant, both the squamous island and the submucosal gland duct were P53 wild type. In another case, a silent point mutation in the second exon of the P16 gene was discovered in an area of Barrett's metaplasia and the same P16 mutation was identified in an adjoining submucosal gland duct. These experiments strongly support the contention that cells within submucosal gland ducts can give rise to both squamous and Barrett's epithelium.

In 1988, Gillen and colleagues reported the results of an insightful experiment [38]. These investigators assigned 25 mongrel dogs either to a control group or to one of four experimental groups. Animals in the four experimental groups underwent circumferential mucosal stripping of two separate 2 cm wide sections of squamous epithelium lining the distal esophagus separated by a 2 cm section of intact squamous epithelium. Animals in three of the four experimental groups then underwent creation of a hiatal hernia while animals in the fourth group did not. Of the animals which underwent hiatal hernia creation, one group was given pentagastrin daily to induce acid secretion, a second group underwent an additional biliary diversion and ligation of the common bile duct to the lesser curvature of the stomach to induce acid secretion and bile reflux, and a third group underwent biliary diversion and ligation of the common bile duct to the lesser curvature of the stomach followed by treatment with cimetidine, an H2 blocker which suppresses acid, twice daily postoperatively to induce exposure to bile salts only. After 3 months, the GEJ and distal esophagus were evaluated in all animals. Animals with a hiatal hernia which received pentagastrin (acid group) or those with a hiatal hernia and biliary diversion (acid and bile salt group) had columnar epithelialization in the lower stripped ring. Regenerating columnar epithelium contained both goblet and parietal cells. Animals with a hiatal hernia and biliary diversion treated with cimetidine (bile salt group) or those without a hiatal hernia (no reflux) reepithelialized only with squamous epithelium. Of the animals with a hiatal hernia treated with pentagastrin, two also developed columnar epithelium in the upper stripped ring. This led to the conclusion that the presence of columnar epithelium in the upper ring beyond an intact squamous barrier ruled out "creeping substitution" from the gastric cardia and that the source of columnar cells was submucosal gland ducts since ducts contiguous with esophageal surface ulcerations were observed.

Another study reported several years later also supported submucosal glands as the source of esophageal epithelial progenitor cells. Li and colleagues performed rectangular-shaped mucosal stripping of the distal esophagus in 12 mongrel dogs, leaving 1 cm of intact squamous epithelium entirely around the stripped area [39]. Sutures were placed at the four corners of the stripped area to demarcate it. The animals underwent creation of a hiatal hernia and then were treated with pentogastrin daily for 3 months. After 3 months, the regenerated epithelium within the boundaries of the sutures was stripped from ten surviving animals and analyzed. Seven animals were found to have columnar epithelium without evidence of squamous islands.

No goblet cells were observed, but in most animals the columnar epithelium was contiguous with ducts of deep submucosal glands. Six of the ten animals then underwent surgical repair of the hiatal hernia followed by treatment with omeprazole for acid suppression. Three months later, the animals were sacrificed and the restripped epithelium within the four suture boundaries was analyzed. All six animals again had columnar metaplasia but now multiple squamous islands were found throughout the metaplastic epithelium. These squamous islands were contiguous with submucosal gland ducts.

More recently, rodents have become the preferred model system for studying Barrett's pathogenesis through surgically induced reflux procedures such as esophagojejunostomy or esophagoduodenostomy. Investigators at Mayo Clinic have even developed a method to use magnets to induce fistula formation between the distal esophagus and small bowel [40]. Rat and mouse esophagi do not contain esophageal submucosal glands [35, 41], and thus in these animal models the source of metaplastic epithelium must be elsewhere.

Circulating Bone Marrow-Derived Stem Cells

In esophageal injury models, bone marrow-derived stem cells can migrate to the esophagus and reconstitute esophageal epithelium. Epperly and colleagues irradiated female C57BL/6 mice with 30 Gray (Gy) to the upper torso, inducing lethal radiation esophagitis, and then injected the female mice with GFP positive male whole bone marrow cells [23]. The female GFP negative mice were sacrificed 14–21 days later and the esophagi evaluated. After 14 days, GFP and Y chromosome positive foci were seen in esophageal squamous epithelium. These GFP positive foci further increased in size after 21 days. The cells were confirmed to be epithelial by hematoxylin and eosin (H&E) staining and pan-CK IHC.

Since the reflux of acid and bile salts injures the esophageal squamous epithelium in patients with GERD, bone marrow-derived cells might also migrate to the esophagus to repair GERD-induced injury. To model this, Sarosi and colleagues irradiated female Sprague-Dawley rats at 6 weeks of age with 900cGy followed by tail vein injection of bone marrow cells obtained from age-matched male rats [42]. Ten days later, the female rats were randomized to either esophagojejunostomy or a sham operation. The esophagi were then harvested and analyzed 8 weeks postoperatively. Four of ten animals who underwent esophagojejunostomy and nine of ten sham-operated animals survived until 8 weeks. Animals that underwent esophagojejunostomy were found to have loss of keratinization, papillary lengthening, basal cell hyperplasia, and mucosal ulceration in addition to intestinal metaplasia while sham-operated animals exhibited none of these features. Fluorescence in situ hybridization (FISH) demonstrated that Y chromosome containing cells gave rise to both squamous and metaplastic columnar epithelium as differentiated by squamous CK14 expression. Further, in sham-operated animals Y chromosome containing cells were also found in female esophageal epithelium,

demonstrating that bone marrow-derived cells can contribute to esophageal epithelium even in the absence of injury.

In another study, Hutchinson and colleagues evaluated bile salt injury in 12 lethally irradiated C57BL/6 mice [43]. These mice subsequently received bone marrow transplants from Gt(ROSA)LacZ mice [44] and underwent esophagojejunostomy. Twelve mice survived until the end of the study and were evaluated after 20 weeks. One-third of the mice were found to have intestinal metaplasia with half of the metaplastic glands expressing β-galactosidase. The β-galactosidase expressing cells were found in groups of 3–4 and never comprised an entire gland. They were shown to be epithelial by costaining with E-cadherin. Interestingly, Kalabis and colleagues reported a similar experiment in FVB/N mice but did not perform esophagojejunostomy. Recipient FVB/N mice were irradiated with 12Gy and given the bone marrow of Gt(ROSA)-enhanced GFP mice. Though there was 95 % engraftment in the bone marrow, no GFP positive cells were found in the esophagus [25]. Esophageal injury, similar to that caused by reflux of bile salts, may be required to cause migration of bone marrow-derived stem cells to the mouse esophagus.

Hutchinson and colleagues also described the development of an esophageal carcinoma in a 45-year-old male who had received a bone marrow transplant at age 35 from his sister for M2 acute myeloid leukemia. He presented with dysphagia and an esophagogastroduodenoscopy (EGD) identified an 8 cm distal esophageal tumor. Biopsy revealed adenosquamous carcinoma, and X/Y chromosome FISH demonstrated that the tumor contained at least 6 % XX, 0Y cells. XX carcinoma cells were intermingled with Y containing cells and colocalized with E-cadherin. It was unclear whether the patient had gastroesophageal reflux or whether his tumor was due to a chronically immunosuppressed state as the patient was reported to have severe graft versus host disease.

These studies raise the possibility that human Barrett's esophagus might originate from a circulating, multipotent bone marrow-derived stem cell. What is important to note is that bone marrow-derived cells did not give rise to complete glands in either rodents on in the case report of the patient with adenosquamous carcinoma. Though bone marrow-derived stem cells can contribute to esophageal epithelium, they may not be the sole source of metaplastic epithelium.

Proximally Shifting Columnar Progenitor Cells

As described earlier, experiments in mongrel dogs supported submucosal gland ducts as the source of Barrett's epithelium. However, prior to those reports Bremner and colleagues came to a different conclusion in 1970 [45]. These investigators divided 35 mongrel dogs into three groups. All animals underwent stripping of the mucosa from the distal 6–10 cm of the esophagus and ending at the GEJ. Two groups of animals then underwent surgical destruction of the lower esophageal sphincter and creation of a hiatal hernia to induce reflux, with one of these groups treated between two and 12 weeks with histamine to enhance acid secretion. In animals that only

underwent mucosal stripping, reepithelialization occurred quickly and was predominantly squamous, though in nine of ten animals columnar epithelium was also found in the distal esophagus. This would be consistent with a wound healing-like mechanism. In animals exposed to reflux, reepithelialization was much slower. Out of 14 animals who did not receive histamine, 12 animals had presence of columnar epithelium and squamous epithelium, with one animal having no squamous epithelium at all. In 11 animals who received histamine, one did not reepithelialize. In half of the remaining ten animals, only columnar epithelium was present. These investigators concluded that reepithelialization can occur with columnar epithelium, that columnar epithelium is favored in the setting of reflux, and this epithelium comes from gastric or junctional epithelium through "creeping substitution." The finding of columnar metaplasia in dogs in the setting of gastroesophageal reflux was confirmed by others several years later [46].

More recent studies in support of the "creeping substitution" hypothesis have utilized mouse genetic models. As discussed before, the mouse esophagus differs from the human esophagus in that it lacks submucosal glands and stromal papillae and its epithelium is keratinized at the luminal surface (Fig. 10.2). Unlike in humans where the normal squamous epithelium-lined esophagus directly meets the columnar epithelium-lined gastric cardia at the GEJ, in mice the squamous epithelium-lined esophagus ends in a pouch at the junction of the squamous epithelium-lined forestomach and columnar-lined glandular stomach. The forestomach is separated from the glandular stomach through the rest of its circumference by a structure known as the limiting ridge (Fig. 10.3). The entire limiting ridge is covered by squamous epithelium, which transitions into columnar epithelium as the limiting ridge meets the glandular stomach. Thus, in humans the SCJ occurs at the GEJ while in mice the SCJ occurs at the distal end of the limiting ridge at the junction of the forestomach and glandular stomach.

Recent interest has focused on the distal limiting ridge as the putative source of Barrett's epithelium. The first of several high profile studies found similarities between forestomach epithelium from $p63^{-/-}$ mouse embryos and Barrett's epithelium as p63 is required for stem cell maintenance in stratified epithelium and is absent in human Barrett's esophagus [47]. At E18, wild-type embryos had forestomachs covered by a stratified squamous epithelium while $p63^{-/-}$ embryos had forestomachs lined with columnar epithelium that secreted mucus. In addition, $p63^{-/-}$ epithelium had higher expression levels of Villin and Agr2, intestinal and mucin associated genes, respectively, that are expressed in human Barrett's esophagus. Using gene expression microarrays (GEM), 17 of the top 50 upregulated genes in the mutant p63 forestomach were found to be upregulated in two separate Barrett's esophagus GEM datasets. Interestingly, the $p63^{-/-}$ forestomach did not express Cdx2, an intestinal transcription factor frequently found in Barrett's epithelium. A time course examining epithelial development of the wild-type forestomach between E13 and E19 demonstrated that the forestomach was initially lined by a Carbonic anhydrase 4 (Car4) expressing monolayered epithelium. As embryogenesis progressed, these Car4 expressing epithelial cells were then pushed toward the lumen by a p63 positive epithelial cell population beginning at E13–14.

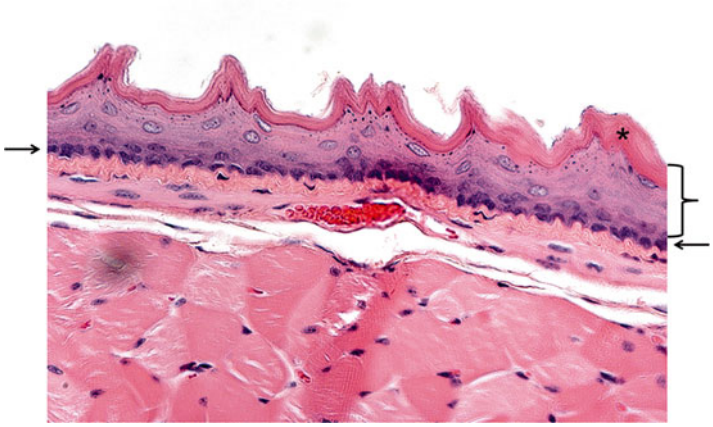

Fig. 10.2 Mouse esophagus (40×). Unlike the human esophagus, the mouse esophagus lacks submucosal glands and stromal papillae. The epithelium is keratinized at the luminal surface (marked with *). Basal cells are found attached to the basement membrane and are indicated by the *arrows*. Suprabasal cells, which are more differentiated, are indicated by the curly brace

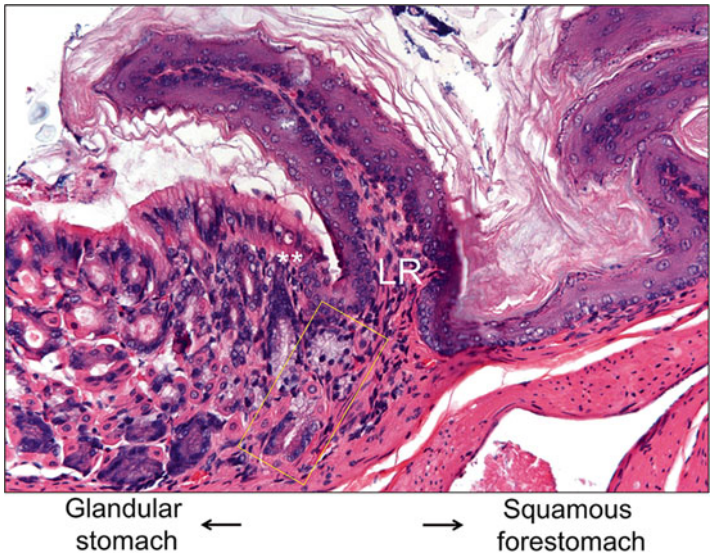

Fig. 10.3 The mouse squamocolumnar junction (20×). The squamous epithelium-lined forestomach (*right*) and the columnar epithelium-lined glandular stomach (*left*) are separated by the squamous epithelium-lined limiting ridge (LR). The transition from stratified squamous to columnar epithelium occurs where the limiting ridge meets the glandular stomach. The first gland of the gastric fundus is indicated by the *box*. The top of the gland reaches the luminal surface in close proximity to the CK7 positive, Dclk1 positive, Lgr5 positive cells (**) as described in Refs. [47, 52, 58]

This led to decreased proliferative capacity (measured by Ki-67 IHC) of the Car4 positive cells as they lost contact with the basement membrane. These investigators from the McKeon lab postulated that in $p63^{-/-}$ embryos, Car4 positive cells remained proliferative and in contact with the basement membrane and gave rise to columnar mucin secreting cells by E18. As embryogenesis continued in the wild-type mouse, Car4 cells expressed CK7 though not all CK7 positive cells expressed Car4. They then tracked CK7 cells during esophageal and forestomach development in wild-type embryos and discovered that this CK7 expressing epithelium was completely sloughed off except for approximately 30 cells which remained at the luminal surface at the SCJ at birth. In association with these remaining CK7 positive cells was an occasional Car4 expressing cell attached to the basement membrane. The remaining CK7 positive cells also expressed Muc4 and survived into adulthood as "residual embryonic cells." Similar CK7, MUC4 positive cells were also found at the luminal surface of the human embryonic and adult SCJ. Induction of injury in CK14 positive squamous epithelium overlying the distal limiting ridge in 3-week-old wild-type mice using Diptheria toxin A (DTA), in an attempt to simulate acid-induced injury, caused CK7 positive epithelium at the SCJ to proximally shift and repopulate the distal limiting ridge. The theory that Barrett's esophagus arises by proximal shifting of residual embryonic cells into voids left by damaged squamous epithelium was put forth. Over 50 years ago, Barrett's epithelium was postulated to represent a persistence of embryonic esophageal epithelium in the distal esophagus that was not replaced during the transition to stratified squamous [48]. Thus, the renewed interest in this concept is clearly warranted.

This intriguing study by Wang and colleagues raises several important questions. First, can the human SCJ population of CK7, MUC4 expressing cells give rise to Barrett's esophagus? The expression of CK7 has been detected in both superficial and deep metaplastic glands in patients with Barrett's esophagus [49]. If so, then the CK7 cells would need to proximally shift into the esophagus and undergo phenotypic and proliferative change (i.e., molecular reprogramming) to form a glandular tissue. Second, do CK7 positive cells and Car4 positive cells share similar characteristics and have the capacity to phenotypically change into intestinal cells (i.e., express Cdx2)? Though the mouse SCJ population of CK7 positive cells was able to proximally shift following injury to adjacent squamous epithelial cells, Car4 cells did not move. These long-term questions remained unanswered because mice could only be followed out to 10 days post-DTA induction due to collateral damage of nearby squamous epithelium. Third, if residual embryonic cells reside superficially at the SCJ, wouldn't they have maximum exposure to acid and bile salts, the physiological components of gastric refluxate? Conceivably as a resident stem cell, CK7 positive cells located at the luminal surface might be more resistant to reflux-induced injury. As the investigators noted in a later review, their work did not directly address how Barrett's metaplasia occurs in surgical rodent models of bile reflux (esophagojejunostomy or esophagoduodenostomy) in which the SCJ region is bypassed [50]. They did suggest that residual embryonic cells in other parts of the GI tract could potentially give rise to Barrett's metaplasia in these surgical models and that the validity of their theory depends on identification of these other populations of residual embryonic cells.

The second study examined the phenotype of mice overexpressing human IL-1β in stratified squamous epithelial cells of the oral cavity, esophagus, and forestomach in which the ED-L2 (an Epstein–Barr virus) promoter is activated [51]. These animals developed a systemic inflammatory reaction as exhibited by splenomegaly; increased serum levels of IL-1β, TNFα, and IL-6; and acute and chronic inflammatory infiltrates in the esophagus and forestomach [52]. At 6 months of age, these mice developed epithelial hyperplasia at the SCJ. By 12–15 months of age, these mice developed columnar metaplasia without goblet cells at the SCJ with Muc5ac, Cdx2, and Tff2 (a marker for spasmolytic polypeptide-expressing metaplasia or SPEM) expressing cells. Between 20 and 22 months, 20 % of the animals developed high-grade dysplasia or intramucosal adenocarcinoma at the SCJ. In adjacent stroma, an increase in myofibroblasts and global hypomethylation was seen, consistent with a stromal role in tumorigenesis. Molecularly, there was increased Wnt (indicated by nuclear β-catenin), Notch, Sonic hedgehog (Shh), Bone morphogenetic protein 4 (Bmp4), and Akt signaling with occasional loss of p16. While acidified water (pH 2.0) did not accelerate the metaplasia time course, adding the unconjugated bile acid deoxycholate to drinking water led to increased inflammatory infiltrates and development of SCJ metaplasia earlier at 9 months and higher degrees of dysplasia at 12 and 15 months with some mice developing tumors at 15 months. Adding the carcinogen N-methyl-N-nitrosurea to deoxycholate led to SCJ tumor development at 12 months. GEM analysis of the mouse SCJ metaplastic lesions in 15-month-old EDL2-IL-1β and 9-month-old EDL2-IL-1β + bile acid-treated mice revealed overlap of 609 genes with human Barrett's esophagus. Finally, Quante and colleagues demonstrated that the metaplasia induced by IL-1β overexpression led to an expansion of Lgr5 and Dclk1 positive stem cells. This is relevant as LGR5 and DCLK1 positive cells have been detected in human Barrett's metaplasia [53, 54]. The location of the Lgr5 cells appeared to overlap with the CK7 positive cell population reported by Wang and colleagues [47].

The third study examined the phenotype of mice overexpressing Bmp4 in CK14 expressing basal squamous epithelium in the esophagus and forestomach. Epithelial Bmp4 overexpression upregulated stromal expression of the Bmp4 inhibitor Noggin, with higher levels of Noggin noted in the proximal esophagus as compared to the forestomach. At 20 weeks, mice were found to have metaplastic glands at the SCJ which expressed phosphorylated Smads 1/5/8, transcription factors that mediate downstream Bmp signaling. A subset of cells in these glands stained for squamous markers such as CK5, CK14, and the squamous stem cell marker p63, whereas other cells stained with columnar markers CK8, CK19, and the intestinal and gastric cardia stem cell marker Lgr5. The glands also contained mucus cells and Tff2 positive cells but did not express Cdx2, Muc2, or Muc5ac, thus resembling cardia-type metaplasia [55]. Twelve weeks after an esophagojejunostomy established with a microsurgical technique using magnets in wild type mice, metaplasia developed at the neo-SCJ in the setting of inflammation. The metaplasia resembled human cardia epithelium with a few Cdx2 expressing cells, but no cells exhibited expression of Muc2. Later at 16 weeks, the metaplastic glands expressed both Cdx2 and Muc2. In vitro experiments further demonstrated that coexpression of Bmp4 and Cdx2 was

required to induce Muc2 expression and that a Smad/Cdx2 transcriptional complex was necessary to transactivate Muc2. These findings suggested that the nonintestinal, cardia type of metaplasia evolved over time to an intestinal type of metaplasia in the setting of reflux induced injury. In fact, indirect evidence supports this in human patients [55–57].

Additional insight into the mouse gastric cardia was provided by a fourth study in which mice null for Smad3, a mediator of TGF-β signaling, developed tumors at the SCJ [58]. At 6 months, these mice had grossly exophytic growths on the lesser curvature of their stomachs. Histologically, metaplastic glands with cells expressing Tff2 were observed just distal to the limiting ridge. At 10 months, these metaplastic gastric glands exhibited dysplastic changes with increased expression of Ki-67 and phosphorylated Stat3, a key mediator of inflammation and cancer. In addition, some cystic structures lined by metaplastic epithelium invaded into submucosal and muscle tissue. In the normal fundus of wild-type mice, these investigators found Dclk1 positive cells in the first gland of the gastric fundus just distal to the limiting ridge, at the junction of squamous and columnar epithelium. In the Smad3$^{-/-}$ mice, Dclk1 positive cells were expanded in their normal location and were seen in invasive and noninvasive metaplastic glands. Thus, the first gland of the gastric fundus may be the origin of gastric metaplasia as well as tumorigenesis in this model. Interestingly, the location of the Dclk1 positive cells appeared to be similar to the Lgr5 cells reported by Quante and colleagues [52].

While Wang and colleagues demonstrated a proximal shift of CK7 positive columnar cells into an area previously inhabited by squamous epithelium, the other studies did not demonstrate movement of metaplastic glandular tissue into the esophagus. This may be because the immediately adjacent proximal squamous epithelium was not injured in the latter studies. It remains to be seen if the metaplastic glands reported in EDL2-IL-1β, CK14-Bmp4, or Smad3$^{-/-}$ mice would proximally shift in the setting of injury to adjacent squamous epithelium.

Transcommitment

Transcommitment, which refers to molecular reprogramming of stem or progenitor cells, is almost certainly required for the development of Barrett's epithelium because some type of phenotypic change is required to generate specialized intestinal metaplasia regardless of the Barrett's esophagus cell or tissue of origin. For example, Barrett's epithelium can contain Paneth cells, enteroendocrine cells, cells that resemble jejunal absorptive cells, and mature goblet cells [59–61], cell types that are found in both gastric and intestinal tissues. Further, proximal shifting of either residual embryonic cells at the SCJ or gastric cardia does not explain how patients who have undergone partial esophagectomy with esophagogastric anastomosis can develop columnar metaplasia in the residual esophagus [62, 63]. In these patients the anastomosis of oxyntic stomach to squamous cervical esophagus removes both the SCJ and gastric cardia [62]. Finally, following ablation of Barrett's

esophagus, reepithelialization by squamous cells is favored in the setting of acid suppression whereas recurrent Barrett's epithelium is favored when acid suppression is inadequate suggesting that the progenitor cell has the capacity to differentiate into either a squamous or columnar cell phenotype depending on the local esophageal environment [64, 65].

Transcommitment could explain how progenitor cells found in esophageal submucosal glands or their ducts give rise to multiple phenotypes. Using endoscopic mucosal resection (EMR) or esophagectomy specimens and mitochondrial DNA mutation analysis in cytochrome c oxidase deficient cells, Nicholson and colleagues demonstrated that esophageal submucosal glands are made up of clonal units [66]. More importantly, they went on to show in a specimen of Barrett's metaplasia from a patient who had undergone ablative therapy that the metaplastic glands and overlying neosquamous epithelium shared the same mitochondrial DNA mutation, suggesting they arose from the same progenitor cell.

Conceptually, an epithelial progenitor cell that gives rise to Barrett's esophagus, regardless of origin, would need to acquire or maintain a columnar phenotype, undergo intestinalization, and secrete mucus as goblet cells are the *sine qua non* of specialized intestinal metaplasia (Fig. 10.4). Switching from a squamous to a columnar phenotype would require the ability to activate transcription factors that

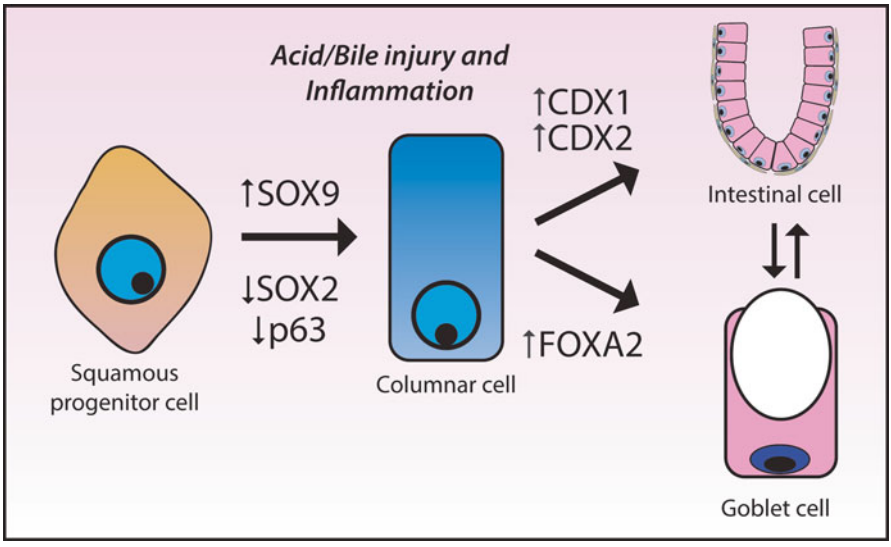

Fig. 10.4 Transcommitment model for Barrett's Esophagus. Following acid and bile injury and/or resultant inflammation, a squamous progenitor cell could upregulate the columnar transcription factor SOX9 and downregulate squamous transcription factors SOX2 and P63 to convert into a columnar cell. This metaplastic columnar cell could then upregulate intestinal transcription factors CDX1 and CDX2 to become an intestinal cell or upregulate the mucin associated transcription factor FOXA2 to become a mucin secreting goblet cell. To become a specialized intestinal metaplastic cell, the intestinalized columnar cell may become an intestinalized goblet cell or the goblet cell could become intestinalized

characterize columnar cells (i.e., Sox9) and/or downregulate transcription factors that characterize squamous cells (i.e., Sox2 and p63) [13, 67]. This would need to be followed by activation of intestinal (i.e., Cdx1 and Cdx2) and mucin associated transcription factors (i.e., Foxa2) [16, 68].

Sox9, a member of the SOX gene family, was first identified in the GI tract within proliferative cells of intestinal crypts as well as Paneth cells [69]. Sox9 is expressed in the CLE during embryogenesis along with CK8 and CK18, but Sox9 expression is lost when the epithelium becomes squamous [13]. To determine whether SOX9 may have functional relevance to the development of Barrett's esophagus, Wang and colleagues performed SOX9 IHC on esophageal tissue microarrays representing 96 esophagectomy cases containing Barrett's esophagus and/or esophageal adenocarcinoma. Nuclear SOX9 was expressed in 100 % of patients with Barrett's epithelium and 85 % of patients with esophageal adenocarcinoma but not in adjacent squamous epithelium [13]. SOX9 was activated in Barrett's epithelium through acid and bile-induced Hedgehog ligand secretion by epithelial cells that in turn activated BMP4 secretion by adjacent stromal cells. This stromal BMP4 then acted back on the epithelium to induce SOX9 expression. Though Sox9 is a Hedgehog target gene in chondrocytes and the skin [70, 71] and it has a distant enhancer region containing a Gli1 binding site [72], SOX9 expression could not be directly induced by Hedgehog pathway activation in human esophageal epithelial cells. Instead, treatment of esophageal squamous HET-1A cells with human recombinant BMP4 or transfection with a constitutively active form of the BMP type I receptor BMPRIA led to increased mRNA expression of *SOX9* by quantitative real-time PCR (qPCR). Overexpression of either SOX9 or constitutively active BMPRIA in HET-1A cells led to expression of columnar CK8/18 [13]. In an in vivo transplant culture system, esophageal epithelial cells from a Shh transgenic mouse induced stromal Bmp4 and epithelial Sox9 expression, establishing that SOX9 is a target of Hedgehog-BMP4 signaling in Barrett's esophagus [13]. Using wild type C57BL/6 mouse esophageal epithelium in the same in vivo transplant culture system, Clemons and colleagues found that retroviral transduction of Sox9 induced expression of columnar CK8 and of the intestinal glycoprotein A33 and changed the stratified squamous epithelium into one to two layers of cuboidal or columnar shaped epithelial cells [73]. In contrast, retroviral transduction of Cdx2 did not alter squamous differentiation or induce either columnar or intestinal gene expression. These data demonstrated that Sox9 expression in esophageal squamous epithelial cells induced markers and morphological changes characteristic of a columnar phenotype.

Sox2, another member of the SOX family of transcription factors, is expressed in the embryonic esophagus where its presumed role is to regulate endoderm differentiation into stratified squamous epithelium [74]. In Sox2 hypomorphic mice, the esophageal epithelium was observed to be thinner, characterized by multilayered columnar cells, and expressed mucin. There was also decreased expression of both p63 and CK14. Sox2 overexpression in the mouse intestine using a conditional Villin promoter led to loss of villi, appearance of p63 expressing basal cells (characteristic of the forestomach and esophagus), and decreased binding of Cdx2

to the promoters of its target genes [75]. A role in squamous differentiation was further supported by the findings of increased SOX2 expression in squamous cancers of the GI tract and lung and that SOX2 and P63 colocalized on genes required for squamous cell carcinoma growth [67, 76, 77]. In addition to its role as a squamous differentiation factor, Sox2 also plays a role in stem cell maintenance. In mice with thymidine kinase (TK) inserted into the endogenous Sox2 locus, treatment with ganciclovir over 2 weeks led to loss of Sox2 expressing esophageal basal cells but maintenance of suprabasal epithelium; these epithelial changes were reversible with a shorter duration of ganciclovir treatment, suggesting that this shorter exposure allowed some Sox2 positive basal cells to survive [78]. Overexpression of Sox2 in the basal epithelium of the mouse esophagus using a conditional CK5 promoter caused basal cell hyperplasia [79]. Combining Sox2 overexpression in esophageal basal epithelial cells with constitutive activation of Stat3 using a Lentiviral construct, led to formation of squamous cell carcinomas. These findings demonstrated that Sox2 is an essential factor for squamous cell differentiation and tissue maintenance in the esophagus. In the normal adult esophagus of both rodents and humans, Sox2 is expressed in the basal cells of the stratified squamous epithelium. In contrast, Sox2 is not expressed in MLE or intestinal metaplasia of the esophagus. Thus, downregulation of Sox2 may be required to reprogram esophageal progenitor cells into Barrett's epithelium [9].

P63 is a member of the P53 family of transcription factors and has six isoforms [80]. Three full length proteins, known as TAp63, contain an amino terminal transactivation domain. Three proteins transcribed from an alternate promoter in the third intron, known as ΔNp63, do not contain the transactivation domain but retain the carboxyl terminal DNA binding domain. Because of alternative splicing at the C-terminus, there are three forms each of TAp63 and ΔNp63 designated as α, β, and γ. Given that mice null for p63 completely lacked stratified squamous epithelium and have esophagi lined by simple columnar epithelium [81], it is likely that downregulation of p63 also plays a role in the formation of Barrett's metaplasia. As described earlier, $p63^{-/-}$ mice developed a Barrett's like metaplasia in the forestomach [47]. Multiple studies have examined P63 expression in esophageal squamous epithelium, esophageal squamous cell carcinoma, Barrett's esophagus, and esophageal adenocarcinoma. Using an antibody against the carboxyl terminus of P63, which recognized all 6 isoforms, one group of investigators reported moderate to strong P63 expression in esophageal squamous epithelium, absent to moderate expression in Barrett's esophagus, and high expression in Barrett's esophagus with high-grade dysplasia and esophageal adenocarcinoma [82]. Other investigators performed IHC with the commonly used 4A4 antibody followed by RT-PCR using primers specific for TAp63 and ΔNp63 [83]. They found that esophageal squamous epithelium and esophageal squamous cell carcinoma strongly expressed P63, while Barrett's esophagus and esophageal adenocarcinoma did not. ΔNp63 was the predominant isoform expressed by esophageal epithelium. Finally, a third group used the 4A4 antibody as well as an antibody that specifically recognized ΔNp63 [84]. They found that ΔNp63 was strongly expressed by esophageal squamous epithelium and esophageal squamous carcinomas while Barrett's epithelium and

esophageal adenocarcinoma rarely expressed P63. Adenocarcinomas that did express P63 expressed the TA isoforms in 63 % of cases. Although these studies had somewhat conflicting results, it appeared that ΔNp63 is the predominant form of p63 in the normal esophagus and that it is required for squamous differentiation. In addition, Barrett's esophagus without dysplasia is not likely to express P63 while adenocarcinomas may weakly express P63, favoring TAp63 isoforms. Another study examined the effect of bile acids and acidified media on P63 expression in primary esophageal epithelial cells and in esophageal squamous cell carcinoma cell lines [85]. It was found that the predominant P63 isoform expressed was ΔNp63α and that ΔNp63α expression could be synergistically repressed with deoxycholic acid and acidic media (pH 5) in a squamous carcinoma cell line. In primary esophageal epithelium, ΔNp63α repression was mostly mediated by the bile salt with minimal additive effect from acidified media. Taken together, these data suggested that bile salts in patients with GERD can suppress expression of ΔNp63 in the squamous-lined esophagus leading to reprogramming of esophageal progenitor cells and the development of Barrett's esophagus.

Following the acquisition and maintenance of a columnar phenotype, the progenitor cell still must undergo intestinalization to generate specialized intestinal metaplasia characterizing Barrett's esophagus. Cdx1 and Cdx2 are members of the caudal related homeobox gene family which are expressed in the intestine [86]. Cdx1 is expressed in the proliferative crypt compartment, while Cdx2 is expressed in the differentiated villus compartment [87]. Cdx1 is expressed in two waves during embryogenesis, initially between E7.5 and 12.5 in the ectoderm and mesoderm. The second wave begins at E12.5 in the gut endoderm and continues through adulthood [87]. Cdx1 knockout mice had anterior homeotic shift of the axial skeleton but no known gut phenotype [88]. Targeting Cdx1 expression to gastric parietal cells using a rat H/K-ATPase promoter, investigators found Cdx1 transgenic mice developed intestinal metaplasia of the gastric epithelium with all four cell types of the adult colon represented including enterocytes, Paneth cells, goblet cells, and enteroendocrine cells [89]. This is consistent with Cdx1 reprogramming columnar progenitors into intestinal columnar cells. *CDX1* mRNA has been found in Barrett's metaplastic tissue, but not in normal esophageal squamous tissue [90]. By bisulfite sequencing, the CDX1 promoter was found to be methylated and silenced in squamous epithelium but demethylated and active in Barrett's epithelium. Treatment of the esophageal squamous cell carcinoma cell line OE21 with 5-azacitadine, a demethylating agent, led to CDX1 expression. NFk-B, TNF-α, and IL-1β (proinflammatory cytokines elevated in reflux esophagitis) were all able to induce CDX1 expression in the colorectal cell line C32. Further, exposure to bile salts or acidified bile salts led to CDX1 expression in C32 cells while exposure to acid alone did not. Investigators in the Kinoshita lab performed esophagojejunostomy in Wistar rats using the Levrat procedure [68]. Seven weeks postoperatively, classic features of reflux esophagitis such as basal cell hyperplasia and papillary lengthening were observed. Cdx1 nuclear staining was seen in squamous epithelium above the anastomosis. At 6 months, columnar metaplasia with goblet cells had arisen and expressed Cdx1. This colocalized with Cdx2 expression within metaplastic Barrett's

epithelium. In vitro, bile salts activated CDX1 promoter luciferase activity in HET-1A and esophageal adenocarcinoma OE33 cells and induced CDX1 protein expression in primary cultured esophageal keratinocytes. Overexpression of Cdx1 in HET-1A cells caused expression of MUC2. Finally, these investigators demonstrated that Cdx1 and Cdx2 can autoregulate their own expression and the expression of each other, establishing a positive feed forward intestinalization loop.

By IHC, CDX2 expression has been found in 100 % of biopsy samples from nondysplastic and dysplastic Barrett's metaplasia and esophageal adenocarcinoma [91, 92]. CDX2 expression has been found in inflamed esophageal squamous epithelium of GERD patients, but not in normal noninflamed esophageal epithelium [93]. In another study, CDX2 expression was found in esophageal squamous epithelium in up to one third of GERD patients with Barrett's esophagus, but not in any samples of esophageal squamous epithelium from GERD patients without Barrett's esophagus [94]. We showed that human esophageal squamous epithelial cells from GERD patients with Barrett's esophagus differentially respond to acid and bile salt exposure by upregulating CDX2 as compared to human esophageal squamous epithelial cells from GERD patients without Barrett's esophagus [17]. These findings suggest that CDX2 may be involved in reprogramming esophageal progenitor cells in those patients with GERD that develop Barrett's esophagus. Like Sox9, Cdx2 is expressed early in the embryonic gut [87]. Unlike Sox9, which is expressed in the embryonic esophagus, Cdx2 is expressed from the duodenum through the gut distally beginning as early as E12.5 [95]. Some insight into its function can be obtained from knockout or overexpression studies in mice. Cdx2 homozygote knockout mice died at E3.5 because of an implantation defect in the trophectoderm [96]. Cdx2 heterozygote mice have been found to develop multiple intestinal adenomatous polyps. Interestingly, keratinized squamous epithelial metaplasia, resembling esophagus or forestomach, was found within these adenomas [96]. Conditional knockout of Cdx2 using the Villin intestinal promoter led to loss of microvilli and intestinal epithelium expressing squamous genes such as p63 and Sox2 [97]. While Cdx2 overexpression in the murine stomach led to intestinal metaplasia [98], Cdx2 overexpression in the murine esophagus using the squamous CK14 promoter did not lead to macroscopic changes of intestinal metaplasia [11]. In vitro, acid induced Cdx2 expression in cultured murine keratinocytes [99]. Additional insight was obtained from CDX2 expression studies in human cells. CDX2 overexpression in vitro in human HET-1A cells led to gland formation [12]. Cdx2's transcriptional targets include intestinal genes such as sucrase-isomaltase [100], MUC2 [101], CK20 [12], Villin [12], and CDX1 [97]. In esophageal EPC2 cells, treatment with the demethylating agent DAC was required for CDX2 to transactivate its target genes. Collectively these data support a simplistic view that CDX2 is insufficient to induce an intestinal phenotype in squamous cells (unless changes in methylation states or other epigenetic changes are induced), while CDX2 can induce intestinal metaplasia in columnar cells. Within the intestine Cdx2 is a major transcriptional activator and thus, Cdx2 loss and resultant loss of its downstream target genes seem to cause a reprogramming of intestinal progenitor cells into squamous cells.

To identify additional proteins that may participate in the pathogenesis of Barrett's esophagus, we performed GEM analysis of RNA isolated from whole esophagus of C57BL/6 embryos at E12.5 versus postnatal day (P)1 pups [16]. These timepoints were chosen to compare gene expression in simple columnar epithelium in the former and stratified squamous epithelium in the latter. From this microarray analysis, we identified another Hedgehog target gene, the transcription factor Foxa2 (Hnf3β), as having a similar esophageal developmental expression pattern as Sox9 [102, 103]. Foxa2 is expressed within the embryonic CLE but not in the adult squamous-lined esophagus [104]. On esophageal tissue microarrays, we found nuclear expression of FOXA2 in Barrett's epithelium but not in normal esophageal squamous epithelium. Further, we found increased expression of *FOXA2* mRNA by qPCR in tissue samples from six cases of Barrett's esophagus. We also found FOXA2 expression by Western blot in telomerase-immortalized Barrett's epithelial cell lines (BAR-T cells) but not in telomerase-immortalized squamous epithelial cell lines from patients with GERD with (NES-BT) or without (NES-GT) Barrett's esophagus [17, 105]. These telomerase immortalized cells showed no signs of tumorigenesis, altered differentiation, or dysregulation of cell proliferation [105–108]. Since NES-BT cells did not express FOXA2, we electroporated them with plasmids containing Shh, Gli1 (a Hedgehog pathway transcriptional activator), and constitutively active BMPRIA. Consistent with prior reports [103, 109], we found that Shh and Gli1 induced *FOXA2* expression. We also found that Hedgehog signaling activated FOXA2 via a Gli dependent enhancer found 3′ to its coding region and that FOXA2 expression in BAR-T cells was decreased following GLI1 siRNA mediated knockdown. In an in vivo transplant culture system, Foxa2 expression was only seen in cultures made from esophageal epithelium from activated Shh transgenic mice. No Foxa2 expression was seen in cultures made from wild type esophageal epithelium. Thus, expression of Shh in mouse esophageal epithelium led to stromal expression of Bmp4 and epithelial expression of Sox9, Foxa2, and columnar CK 8/18 in our in vivo transplant culture system [13, 16]. Foxa2 has been demonstrated to transcriptionally regulate expression of MUC2, the mucin specifically expressed by intestinal epithelium and found in Barrett's esophagus [110, 111]. Proper processing of mature MUC2 protein is regulated by AGR2, a protein disulfide isomerase localized to the endoplasmic reticulum [112]. Analysis of Agr2 knockout mice revealed that they have decreased intestinal mucus and lack morphologically normal goblet cells [113]. As expected, these mice lacked mature Muc2 protein but express *Muc2* transcript within their intestine. We examined expression of MUC2 mRNA and protein in NES-B3T and NES-B10T cells and found these cells expressed little to no *MUC2* mRNA and no protein. Overexpression of FOXA2 in both squamous cell lines led to expression of both MUC2 mRNA and protein. Further, we found that FOXA2 expression in NES-B3T and NES-B10T cells led to expression of AGR2 mRNA and protein. siRNA mediated knockdown of FOXA2 in both BAR-T and BAR-10T cells led to decreased expression of AGR2 mRNA and protein. Together, these data suggested that FOXA2 induced production of intestinal mucus. It does this through presumed transcriptional regulation of MUC2 itself and of AGR2, which is required for proper processing of the MUC2 protein. Though FOXA2 expression led to MUC2 protein expression, the cells

did not acquire a full goblet cell phenotype. It is likely that other factors may be required in addition to FOXA2 to induce a goblet cell phenotype. Based on data from esophageal development, these other factors could include downregulation of SOX2 and P63. Similar to Noggin null mice, in which Bmp4 signaling is unopposed, Sox2 null or p63 null mouse embryos have esophagi with columnar epithelium containing goblet-like cells [47, 74]. Notch pathway modulation may also be required as treatment with gamma secretase inhibitors and the resultant loss of Notch signaling in a surgical model of reflux esophagitis and Barrett's metaplasia led to almost a complete conversion of metaplastic epithelial cells to differentiated goblet cells [114]. Furthermore, the loss of Notch signaling would increase expression of ATOH1, a Notch pathway component which can also regulate MUC2 [115].

Conclusions

Based on available data generated from human patients, dog and rodent models of surgically induced reflux esophagitis, and various cell culture systems, it can be concluded that cells that give rise to Barrett's esophagus can come from multiple tissue sources and most likely undergo transcommitment. Transdifferentiation is unlikely because a nonproliferating differentiated squamous cell could not sustain Barrett's esophagus tissue indefinitely and full phenotypic conversion of a cultured squamous cell has not yet been demonstrated. Esophageal squamous epithelial progenitor cells that retain the embryonic capacity to switch between squamous and columnar phenotype must still undergo molecular reprogramming to generate specialized intestinal metaplasia. Submucosal glands or their ducts have been shown to be contiguous with normal squamous, Barrett's, MLE, and regenerating neosquamous epithelium in human patients and in dogs which have undergone reflux surgery. More convincingly, mutational analysis of P53 and P16 has shown that the same mutation present in a submucosal gland duct is also present in either overlying Barrett's or squamous epithelium. Progenitor cells in the submucosal glands or their ducts would be native to the esophagus, but would have to move out of the glands and ducts into the esophageal epithelium, and undergo molecular reprogramming to give rise to specialized intestinal metaplasia. Circulating bone marrow-derived stem cells have been shown to migrate to the esophagus and regenerate epithelium following injury induced by radiation, surgically induced reflux, or bone marrow transplant preparative regimens in mice, rats, and humans. Residual embryonic cells at the SCJ in mice have been shown to proximally shift to repair immediately adjacent squamous epithelium injured with DTA. A similar mechanism is supported by proximal shifting of gastric cardia cells giving rise to columnar epithelium in dogs as reported by Bremner and colleagues and suggested by the EDL2-IL-1β mouse model. Circulating bone marrow-derived cells, residual embryonic cells at the SCJ, and gastric cardia cells would all have to undergo molecular reprogramming to give rise to specialized intestinal metaplasia. In human patients, data exists to support each of the currently proposed hypotheses and as of yet none can be completely excluded in an individual patient.

Objections to some of these possible sources of Barrett's esophagus include lack of submucosal glands in rodents that develop Barrett's metaplasia from surgically induced reflux, the belief that bone marrow-derived cells do not migrate to the esophagus in the absence of major injury or the finding that they only partially contribute to glands, and the absence of definitive evidence in humans of either transdifferentiation of squamous epithelium or proximal shifting of SCJ or gastric cardia cells. Moreover, these data suggest that sources for the cell or tissue of origin of Barrett's esophagus may vary depending on the species. For example, there is great interest in recently reported transgenic and knockout mouse models and the resulting metaplasia found at the SCJ in many of these mice. Further studies will likely show that the first gland of the gastric fundus, found at the junction of the forestomach and glandular stomach, serves as a reservoir of multipotent progenitors in mice similar to esophageal submucosal glands or ducts in humans that can give rise to both squamous and columnar epithelium. This notion is supported by the finding of MLE, characterized by both squamous and columnar-like cells in CK14-Bmp4 mice.

We speculate that human Barrett's metaplasia can arise from progenitor cells that originate in the esophagus, circulate in the bloodstream, or proximally shift from the SCJ or gastric cardia to fill in voids left by injured squamous epithelium. Regardless of their origin, these progenitors must then undergo transcommitment through molecular reprogramming of the expression levels of different combinations of transcription factors to give rise to the specialized intestinal metaplasia characteristic of Barrett's esophagus. Most likely, progenitor cells give rise initially to epithelial cells with biphenotypic potential (such as seen in MLE), followed by columnar differentiation, intestinalization, and for some mucus differentiation. Sequential activation or knockdown of a logical sequence of transcription factors in human cells in novel cell culture systems or in the appropriate animal model in the future may shed further insight into how Barrett's metaplasia develops in patients with GERD.Funding This work was funded by the US National Institutes of Health (R01-DK097340 to D.H.W. and R01-DK63621 to R.F.S.) and by the Office of Research and Development, US Department of Veterans Affairs (I01-BX001061 and I01-BX002666 to R.F.S.).

References

1. Spechler SJ, Souza RF. Barrett's esophagus. N Engl J Med. 2014;371(9):836–45.
2. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ. American Gastroenterological Association technical review on the management of Barrett's esophagus. Gastroenterology. 2011;140(3):e18–52. quiz e13.
3. Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D. The molecular basis of transdifferentiation. J Cell Mol Med. 2005;9(3):569–82.
4. Cotton CC, Wolf WA, Pasricha S, Li N, Madanick RD, Spacek MB, Ferrell K, Dellon ES, Shaheen NJ. Recurrent intestinal metaplasia after radiofrequency ablation for Barrett's esophagus: endoscopic findings and anatomic location. Gastrointest Endosc. 2015;81(6):1362–9.

5. Shields HM, Zwas F, Antonioli DA, Doos WG, Kim S, Spechler SJ. Detection by scanning electron microscopy of a distinctive esophageal surface cell at the junction of squamous and Barrett's epithelium. Dig Dis Sci. 1993;38(1):97–108.
6. Sawhney RA, Shields HM, Allan CH, Boch JA, Trier JS, Antonioli DA. Morphological characterization of the squamocolumnar junction of the esophagus in patients with and without Barrett's epithelium. Dig Dis Sci. 1996;41(6):1088–98.
7. Boch JA, Shields HM, Antonioli DA, Zwas F, Sawhney RA, Trier JS. Distribution of cytokeratin markers in Barrett's specialized columnar epithelium. Gastroenterology. 1997;112(3):760–5.
8. Glickman JN, Chen YY, Wang HH, Antonioli DA, Odze RD. Phenotypic characteristics of a distinctive multilayered epithelium suggests that it is a precursor in the development of Barrett's esophagus. Am J Surg Pathol. 2001;25(5):569–78.
9. Chen X, Qin R, Liu B, Ma Y, Su Y, Yang CS, Glickman JN, Odze RD, Shaheen NJ. Multilayered epithelium in a rat model and human Barrett's esophagus: similar expression patterns of transcription factors and differentiation markers. BMC Gastroenterol. 2008;8:1.
10. Su Y, Chen X, Klein M, Fang M, Wang S, Yang CS, Goyal RK. Phenotype of columnar-lined esophagus in rats with esophagogastroduodenal anastomosis: similarity to human Barrett's esophagus. Lab Invest. 2004;84(6):753–65.
11. Kong J, Crissey MA, Funakoshi S, Kreindler JL, Lynch JP. Ectopic Cdx2 expression in murine esophagus models an intermediate stage in the emergence of Barrett's esophagus. PLoS One. 2011;6(4):e18280.
12. Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, et al. Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis. 2007;28(2):488–96.
13. Wang DH, Clemons NJ, Miyashita T, Dupuy AJ, Zhang W, Szczepny A, Corcoran-Schwartz IM, Wilburn DL, Montgomery EA, Wang JS, et al. Aberrant epithelial-mesenchymal Hedgehog signaling characterizes Barrett's metaplasia. Gastroenterology. 2010;138(5):1810–22.
14. Stairs DB, Nakagawa H, Klein-Szanto A, Mitchell SD, Silberg DG, Tobias JW, Lynch JP, Rustgi AK. Cdx1 and c-Myc foster the initiation of transdifferentiation of the normal esophageal squamous epithelium toward Barrett's esophagus. PLoS One. 2008;3(10):e3534.
15. Kong J, Nakagawa H, Isariyawongse BK, Funakoshi S, Silberg DG, Rustgi AK, Lynch JP. Induction of intestinalization in human esophageal keratinocytes is a multistep process. Carcinogenesis. 2009;30(1):122–30.
16. Wang DH, Tiwari A, Kim ME, Clemons NJ, Regmi NL, Hodges WA, Berman DM, Montgomery EA, Watkins DN, Zhang X, et al. Hedgehog signaling regulates FOXA2 in esophageal embryogenesis and Barrett's metaplasia. J Clin Invest. 2014;124(9):3767–80.
17. Huo X, Zhang HY, Zhang XI, Lynch JP, Strauch ED, Wang JY, Melton SD, Genta RM, Wang DH, Spechler SJ, et al. Acid and bile salt-induced CDX2 expression differs in esophageal squamous cells from patients with and without Barrett's esophagus. Gastroenterology. 2010;139(1):194–203. e1.
18. De Hertogh G, Van Eyken P, Ectors N, Geboes K. On the origin of cardiac mucosa: a histological and immunohistochemical study of cytokeratin expression patterns in the developing esophagogastric junction region and stomach. World J Gastroenterol. 2005;11(29):4490–6.
19. DeNardi FG, Riddell RH. The normal esophagus. Am J Surg Pathol. 1991;15(3):296–309.
20. Raymond C, Anne V, Millane G. Development of esophageal epithelium in the fetal and neonatal mouse. Anat Rec. 1991;230(2):225–34.
21. Duan H, Gao F, Li S, Nagata T. Postnatal development and aging of esophageal epithelium in mouse: a light and electron microscopic radioautographic study. Cell Mol Biol (Noisy-le-Grand). 1993;39(3):309–16.
22. Yu WY, Slack JM, Tosh D. Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus. Dev Biol. 2005;284(1):157–70.
23. Epperly MW, Guo H, Shen H, Niu Y, Zhang X, Jefferson M, Sikora CA, Greenberger JS. Bone marrow origin of cells with capacity for homing and differentiation to esophageal squamous epithelium. Radiat Res. 2004;162(3):233–40.

24. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183(4):1797–806.
25. Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs DB, Figueiredo JL, Mahmood U, Diehl JA, Herlyn M, et al. A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. J Clin Invest. 2008;118(12):3860–9.
26. Croagh D, Phillips WA, Redvers R, Thomas RJ, Kaur P. Identification of candidate murine esophageal stem cells using a combination of cell kinetic studies and cell surface markers. Stem Cells. 2007;25(2):313–8.
27. DeWard AD, Cramer J, Lagasse E. Cellular heterogeneity in the mouse esophagus implicates the presence of a nonquiescent epithelial stem cell population. Cell Rep. 2014;9(2):701–11.
28. Little MH, Takasato M. Generating a self-organizing kidney from pluripotent cells. Curr Opin Organ Transplant. 2015;20(2):178–86.
29. Doupe DP, Alcolea MP, Roshan A, Zhang G, Klein AM, Simons BD, Jones PH. A single progenitor population switches behavior to maintain and repair esophageal epithelium. Science. 2012;337(6098):1091–3.
30. Ireland H, Kemp R, Houghton C, Howard L, Clarke AR, Sansom OJ, Winton DJ. Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. Gastroenterology. 2004;126(5):1236–46.
31. Seery JP, Watt FM. Asymmetric stem-cell divisions define the architecture of human oesophageal epithelium. Curr Biol. 2000;10(22):1447–50.
32. Barbera M, di Pietro M, Walker E, Brierley C, MacRae S, Simons BD, Jones PH, Stingl J, Fitzgerald RC. The human squamous oesophagus has widespread capacity for clonal expansion from cells at diverse stages of differentiation. Gut. 2015;64(1):11–9.
33. Pan Q, Nicholson AM, Barr H, Harrison LA, Wilson GD, Burkert J, Jeffery R, Alison MR, Looijenga L, Lin WR, et al. Identification of lineage-uncommitted, long-lived, label-retaining cells in healthy human esophagus and stomach, and in metaplastic esophagus. Gastroenterology. 2013;144(4):761–70.
34. Lorinc E, Oberg S. Submucosal glands in the columnar-lined oesophagus: evidence of an association with metaplasia and neosquamous epithelium. Histopathology. 2012;61(1):53–8.
35. Long JD, Orlando RC. Esophageal submucosal glands: structure and function. Am J Gastroenterol. 1999;94(10):2818–24.
36. Coad RA, Woodman AC, Warner PJ, Barr H, Wright NA, Shepherd NA. On the histogenesis of Barrett's oesophagus and its associated squamous islands: a three-dimensional study of their morphological relationship with native oesophageal gland ducts. J Pathol. 2005;206(4):388–94.
37. Leedham SJ, Preston SL, McDonald SA, Elia G, Bhandari P, Poller D, Harrison R, Novelli MR, Jankowski JA, Wright NA. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. Gut. 2008;57(8):1041–8.
38. Gillen P, Keeling P, Byrne PJ, West AB, Hennessy TP. Experimental columnar metaplasia in the canine oesophagus. Br J Surg. 1988;75(2):113–5.
39. Li H, Walsh TN, O'Dowd G, Gillen P, Byrne PJ, Hennessy TP. Mechanisms of columnar metaplasia and squamous regeneration in experimental Barrett's esophagus. Surgery. 1994;115(2):176–81.
40. Mari L, Milano F, Parikh K, Straub D, Everts V, Hoeben KK, Fockens P, Buttar NS, Krishnadath KK. A pSMAD/CDX2 complex is essential for the intestinalization of epithelial metaplasia. Cell Rep. 2014;7(4):1197–210.
41. Rishniw M, Rodriguez P, Que J, Burke ZD, Tosh D, Chen H, Chen X. Molecular aspects of esophageal development. Ann N Y Acad Sci. 2011;1232:309–15.
42. Sarosi G, Brown G, Jaiswal K, Feagins LA, Lee E, Crook TW, Souza RF, Zou YS, Shay JW, Spechler SJ. Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus. Dis Esophagus. 2008;21(1):43–50.
43. Hutchinson L, Stenstrom B, Chen D, Piperdi B, Levey S, Lyle S, Wang TC, Houghton J. Human Barrett's adenocarcinoma of the esophagus, associated myofibroblasts, and

endothelium can arise from bone marrow-derived cells after allogeneic stem cell transplant. Stem Cells Dev. 2011;20(1):11–7.
44. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21(1):70–1.
45. Bremner CG, Lynch VP, Ellis Jr FH. Barrett's esophagus: congenital or acquired? An experimental study of esophageal mucosal regeneration in the dog. Surgery. 1970;68(1):209–16.
46. Pollara WM, Cecconello I, Zilberstein B, Iria K, Pinotti HW. Regeneration of esophageal epithelium in the presence of gastroesophageal reflux. Arq Gastroenterol. 1983;20(2):53–9.
47. Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R, Vincent M, Lu X, Bellizzi AM, Ho KY, et al. Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell. 2011;145(7):1023–35.
48. Goldman MC, Beckman RC. Barrett syndrome. Case report with discussion about concepts of pathogenesis. Gastroenterology. 1960;39:104–10.
49. Ormsby AH, Goldblum JR, Rice TW, Richter JE, Falk GW, Vaezi MF, Gramlich TL. Cytokeratin subsets can reliably distinguish Barrett's esophagus from intestinal metaplasia of the stomach. Hum Pathol. 1999;30(3):288–94.
50. Xian W, Ho KY, Crum CP, McKeon F. Cellular origin of Barrett's esophagus: controversy and therapeutic implications. Gastroenterology. 2012;142(7):1424–30.
51. Nakagawa H, Wang TC, Zukerberg L, Odze R, Togawa K, May GH, Wilson J, Rustgi AK. The targeting of the cyclin D1 oncogene by an Epstein-Barr virus promoter in transgenic mice causes dysplasia in the tongue, esophagus and forestomach. Oncogene. 1997;14(10):1185–90.
52. Quante M, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, Lee Y, Friedman R, Asfaha S, Dubeykovskaya Z, et al. Bile Acid and inflammation activate gastric cardia stem cells in a mouse model of barrett-like metaplasia. Cancer Cell. 2012;21(1):36–51.
53. Becker L, Huang Q, Mashimo H. Lgr5, an intestinal stem cell marker, is abnormally expressed in Barrett's esophagus and esophageal adenocarcinoma. Dis Esophagus. 2010;23(2):168–74.
54. Vega KJ, May R, Sureban SM, Lightfoot SA, Qu D, Reed A, Weygant N, Ramanujam R, Souza R, Madhoun M, et al. Identification of the putative intestinal stem cell marker doublecortin and CaM kinase-like-1 in Barrett's esophagus and esophageal adenocarcinoma. J Gastroenterol Hepatol. 2012;27(4):773–80.
55. Hahn HP, Blount PL, Ayub K, Das KM, Souza R, Spechler S, Odze RD. Intestinal differentiation in metaplastic, nongoblet columnar epithelium in the esophagus. Am J Surg Pathol. 2009;33(7):1006–15.
56. Chaves P, Pereira AD, Cruz C, Suspiro A, Mendes de Almeida JC, Leitao CN, Soares J. Recurrent columnar-lined esophageal segments—study of the phenotypic characteristics using intestinal markers. Dis Esophagus. 2002;15(4):282–6.
57. Dias Pereira A, Chaves P. Columnar-lined oesophagus without intestinal metaplasia: results from a cohort with a mean follow-up of 7 years. Aliment Pharmacol Ther. 2012;36(3):282–9.
58. Nam KT, O'Neal R, Lee YS, Lee YC, Coffey RJ, Goldenring JR. Gastric tumor development in Smad3-deficient mice initiates from forestomach/glandular transition zone along the lesser curvature. Lab Invest. 2012;92(6):883–95.
59. Schreiber DS, Apstein M, Hermos JA. Paneth cells in Barrett's esophagus. Gastroenterology. 1978;74(6):1302–4.
60. Griffin M, Sweeney EC. The relationship of endocrine cells, dysplasia and carcinoembryonic antigen in Barrett's mucosa to adenocarcinoma of the oesophagus. Histopathology. 1987;11(1):53–62.
61. Levine DS, Rubin CE, Reid BJ, Haggitt RC. Specialized metaplastic columnar epithelium in Barrett's esophagus. A comparative transmission electron microscopic study. Lab Invest. 1989;60(3):418–32.
62. Lord RV, Wickramasinghe K, Johansson JJ, Demeester SR, Brabender J, Demeester TR. Cardiac mucosa in the remnant esophagus after esophagectomy is an acquired epithelium with Barrett's-like features. Surgery. 2004;136(3):633–40.

63. Oberg S, Johansson J, Wenner J, Walther B. Metaplastic columnar mucosa in the cervical esophagus after esophagectomy. Ann Surg. 2002;235(3):338–45.
64. Berenson MM, Johnson TD, Markowitz NR, Buchi KN, Samowitz WS. Restoration of squamous mucosa after ablation of Barrett's esophageal epithelium. Gastroenterology. 1993;104(6):1686–91.
65. Brandt LJ, Blansky RL, Kauvar DR. Repeat laser therapy of recurrent Barrett's epithelium: success with anacidity. Gastrointest Endosc. 1995;41(3):267.
66. Nicholson AM, Graham TA, Simpson A, Humphries A, Burch N, Rodriguez-Justo M, Novelli M, Harrison R, Wright NA, McDonald SA, et al. Barrett's metaplasia glands are clonal, contain multiple stem cells and share a common squamous progenitor. Gut. 2012;61(10):1380–9.
67. Watanabe H, Ma Q, Peng S, Adelmant G, Swain D, Song W, Fox C, Francis JM, Pedamallu CS, DeLuca DS, et al. SOX2 and p63 colocalize at genetic loci in squamous cell carcinomas. J Clin Invest. 2014;124(4):1636–45.
68. Kazumori H, Ishihara S, Kinoshita Y. Roles of caudal-related homeobox gene Cdx1 in oesophageal epithelial cells in Barrett's epithelium development. Gut. 2009;58(5):620–8.
69. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, Jay P. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol. 2004;166(1):37–47.
70. Vidal VP, Chaboissier MC, Lutzkendorf S, Cotsarelis G, Mill P, Hui CC, Ortonne N, Ortonne JP, Schedl A. Sox9 is essential for outer root sheath differentiation and the formation of the hair stem cell compartment. Curr Biol. 2005;15(15):1340–51.
71. Tavella S, Biticchi R, Schito A, Minina E, Di Martino D, Pagano A, Vortkamp A, Horton WA, Cancedda R, Garofalo S. Targeted expression of SHH affects chondrocyte differentiation, growth plate organization, and Sox9 expression. J Bone Miner Res. 2004;19(10):1678–88.
72. Bien-Willner GA, Stankiewicz P, Lupski JR. SOX9cre1, a cis-acting regulatory element located 1.1 Mb upstream of SOX9, mediates its enhancement through the SHH pathway. Hum Mol Genet. 2007;16(10):1143–56.
73. Clemons NJ, Wang DH, Croagh D, Tikoo A, Fennell CM, Murone C, Scott AM, Watkins DN, Phillips WA. Sox9 drives columnar differentiation of esophageal squamous epithelium: a possible role in the pathogenesis of Barrett's esophagus. Am J Physiol Gastrointest Liver Physiol. 2012;303(12):G1335–46.
74. Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrisey EE, Taranova O, Pevny LH, Hogan BL. Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. Development. 2007;134(13):2521–31.
75. Raghoebir L, Bakker ER, Mills JC, Swagemakers S, Kempen MB, Munck AB, Driegen S, Meijer D, Grosveld F, Tibboel D, et al. SOX2 redirects the developmental fate of the intestinal epithelium toward a premature gastric phenotype. J Mol Cell Biol. 2012;4(6):377–85.
76. Long KB, Hornick JL. SOX2 is highly expressed in squamous cell carcinomas of the gastrointestinal tract. Hum Pathol. 2009;40(12):1768–73.
77. Bass AJ, Watanabe H, Mermel CH, Yu S, Perner S, Verhaak RG, Kim SY, Wardwell L, Tamayo P, Gat-Viks I, et al. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nat Genet. 2009;41(11):1238–42.
78. Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell. 2011;9(4):317–29.
79. Liu K, Jiang M, Lu Y, Chen H, Sun J, Wu S, Ku WY, Nakagawa H, Kita Y, Natsugoe S, et al. Sox2 cooperates with inflammation-mediated Stat3 activation in the malignant transformation of foregut basal progenitor cells. Cell Stem Cell. 2013;12(3):304–15.
80. Barbieri CE, Pietenpol JA. p63 and epithelial biology. Exp Cell Res. 2006;312(6):695–706.
81. Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM. Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. Am J Physiol Cell Physiol. 2004;287(1):C171–81.
82. Hall PA, Woodman AC, Campbell SJ, Shepherd NA. Expression of the p53 homologue p63alpha and DeltaNp63alpha in the neoplastic sequence of Barrett's oesophagus: correlation with morphology and p53 protein. Gut. 2001;49(5):618–23.

83. Glickman JN, Yang A, Shahsafaei A, McKeon F, Odze RD. Expression of p53-related protein p63 in the gastrointestinal tract and in esophageal metaplastic and neoplastic disorders. Hum Pathol. 2001;32(11):1157–65.
84. Geddert H, Kiel S, Heep HJ, Gabbert HE, Sarbia M. The role of p63 and deltaNp63 (p40) protein expression and gene amplification in esophageal carcinogenesis. Hum Pathol. 2003;34(9):850–6.
85. Roman S, Petre A, Thepot A, Hautefeuille A, Scoazec JY, Mion F, Hainaut P. Downregulation of p63 upon exposure to bile salts and acid in normal and cancer esophageal cells in culture. Am J Physiol Gastrointest Liver Physiol. 2007;293(1):G45–53.
86. Guo RJ, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. Cancer Biol Ther. 2004;3(7):593–601.
87. Silberg DG, Swain GP, Suh ER, Traber PG. Cdx1 and cdx2 expression during intestinal development. Gastroenterology. 2000;119(4):961–71.
88. Subramanian V, Meyer BI, Gruss P. Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. Cell. 1995;83(4):641–53.
89. Mutoh H, Sakurai S, Satoh K, Osawa H, Hakamata Y, Takeuchi T, Sugano K. Cdx1 induced intestinal metaplasia in the transgenic mouse stomach: comparative study with Cdx2 transgenic mice. Gut. 2004;53(10):1416–23.
90. Wong NA, Wilding J, Bartlett S, Liu Y, Warren BF, Piris J, Maynard N, Marshall R, Bodmer WF. CDX1 is an important molecular mediator of Barrett's metaplasia. Proc Natl Acad Sci U S A. 2005;102(21):7565–70.
91. Groisman GM, Amar M, Meir A. Expression of the intestinal marker Cdx2 in the columnar-lined esophagus with and without intestinal (Barrett's) metaplasia. Mod Pathol. 2004;17(10):1282–8.
92. Phillips RW, Frierson Jr HF, Moskaluk CA. Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. Am J Surg Pathol. 2003;27(11):1442–7.
93. Eda A, Osawa H, Satoh K, Yanaka I, Kihira K, Ishino Y, Mutoh H, Sugano K. Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. J Gastroenterol. 2003;38(1):14–22.
94. Moons LM, Bax DA, Kuipers EJ, Van Dekken H, Haringsma J, Van Vliet AH, Siersema PD, Kusters JG. The homeodomain protein CDX2 is an early marker of Barrett's oesophagus. J Clin Pathol. 2004;57(10):1063–8.
95. Beck F, Erler T, Russell A, James R. Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev Dyn. 1995;204(3):219–27.
96. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature. 1997;386(6620):84–7.
97. Gao N, White P, Kaestner KH. Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. Dev Cell. 2009;16(4):588–99.
98. Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, Honda S, Kawata H, Kihira K, Sugano K. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of Helicobacter pylori. Helicobacter. 2002;7(3):192–8.
99. Marchetti M, Caliot E, Pringault E. Chronic acid exposure leads to activation of the cdx2 intestinal homeobox gene in a long-term culture of mouse esophageal keratinocytes. J Cell Sci. 2003;116(Pt 8):1429–36.
100. Boudreau F, Rings EH, van Wering HM, Kim RK, Swain GP, Krasinski SD, Moffett J, Grand RJ, Suh ER, Traber PG. Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. J Biol Chem. 2002;277(35):31909–17.
101. Guo M, House MG, Suzuki H, Ye Y, Brock MV, Lu F, Liu Z, Rustgi AK, Herman JG. Epigenetic silencing of CDX2 is a feature of squamous esophageal cancer. Int J Cancer. 2007;121(6):1219–26.
102. van den Brink GR, Hardwick JC, Tytgat GN, Brink MA, Ten Kate FJ, Van Deventer SJ, Peppelenbosch MP. Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. Gastroenterology. 2001;121(2):317–28.

103. Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development. 1997;124(7):1313–22.
104. Besnard V, Wert SE, Hull WM, Whitsett JA. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expr Patterns. 2004;5(2):193–208.
105. Jaiswal KR, Morales CP, Feagins LA, Gandia KG, Zhang X, Zhang HY, Hormi-Carver K, Shen Y, Elder F, Ramirez RD, et al. Characterization of telomerase-immortalized, non-neoplastic, human Barrett's cell line (BAR-T). Dis Esophagus. 2007;20(3):256–64.
106. Harley CB. Telomerase is not an oncogene. Oncogene. 2002;21(4):494–502.
107. Morales CP, Gandia KG, Ramirez RD, Wright WE, Shay JW, Spechler SJ. Characterisation of telomerase immortalised normal human oesophageal squamous cells. Gut. 2003;52(3):327–33.
108. Zhang HY, Zhang X, Chen X, Thomas D, Hormi-Carver K, Elder F, Spechler SJ, Souza RF. Differences in activity and phosphorylation of MAPK enzymes in esophageal squamous cells of GERD patients with and without Barrett's esophagus. Am J Physiol Gastrointest Liver Physiol. 2008;295(3):G470–8.
109. Lynn FC, Smith SB, Wilson ME, Yang KY, Nekrep N, German MS. Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proc Natl Acad Sci U S A. 2007;104(25):10500–5.
110. van der Sluis M, Vincent A, Bouma J, Korteland-Van Male A, van Goudoever JB, Renes IB, Van Seuningen I. Forkhead box transcription factors Foxa1 and Foxa2 are important regulators of Muc2 mucin expression in intestinal epithelial cells. Biochem Biophys Res Commun. 2008;369(4):1108–13.
111. Ye DZ, Kaestner KH. Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. Gastroenterology. 2009;137(6):2052–62.
112. Park SW, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, Killeen N, Erle DJ. The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. Proc Natl Acad Sci U S A. 2009;106(17):6950–5.
113. Zhao F, Edwards R, Dizon D, Afrasiabi K, Mastroianni JR, Geyfman M, Ouellette AJ, Andersen B, Lipkin SM. Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2−/− mice. Dev Biol. 2010;338(2):270–9.
114. Menke V, van Es JH, de Lau W, van den Born M, Kuipers EJ, Siersema PD, de Bruin RW, Kusters JG, Clevers H. Conversion of metaplastic Barrett's epithelium into post-mitotic goblet cells by gamma-secretase inhibition. Dis Model Mech. 2010;3(1–2):104–10.
115. Tamagawa Y, Ishimura N, Uno G, Yuki T, Kazumori H, Ishihara S, Amano Y, Kinoshita Y. Notch signaling pathway and Cdx2 expression in the development of Barrett's esophagus. Lab Invest. 2012;92(6):896–909.

Chapter 11
Studying Cancer Evolution in Barrett's Esophagus and Esophageal Adenocarcinoma

Thomas G. Paulson

Introduction

The concept that cancers evolve through sequential selection of genetically abnormal cell populations has slowly gained acceptance since Nowell outlined his synthesis of clonal evolution in cancer in 1976 [1]. Perhaps unfortunately, the publication of Nowell's paper coincided with the development of Sanger sequencing in 1977 [2] and the advent of recombinant DNA techniques in the 1970s and 1980s which wrested attention away from the study of how cancers evolve to a more gene-centric approach to cancer research. A recent PubMed search for articles containing "gene" and "cancer" in the title generated over 18,500 results, whereas searching for "evolution" and "cancer" returns only 1000, highlighting the importance placed on roles individual genes are thought to play in carcinogenesis. At the current time, the role of evolution in the development of cancer resembles our understanding of dark matter; there is overall agreement that it exists and has a critical role, but direct visualization of it and the exact details of what role it plays remain elusive. While a full examination of the reasons why characterizing the evolutionary processes involved in the development of cancer have been slow to be adopted is beyond the scope of this review (see [3]), for a review of this topic), we will present a brief overview of how recent advances in the analyses of cancer genomes have advanced (and potentially hindered) our understanding of how genomes evolve during the development of cancer, and describe in more detail recent work in Barrett's esophagus (BE) that highlight some of the practical considerations involved in examining these evolutionary processes in vivo during progression to esophageal adenocarcinoma (EA).

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Cancer Models in the Age of Genome Sequencing

The concept that the evolution of cancer proceeds through the stepwise accumulation of genetic alterations in a predictable, linear manner has existed for many years. Indeed, this has been the paradigm taught in medical schools for decades [4], and the linear model was formalized in analyses of the frequencies of specific genetic alterations present at different stages of neoplastic progression (e.g., see [5, 6]). A linear model of carcinogenesis is attractive in that it fits with phenotypic observations of changes in cell and tissue structure (e.g., normal colon to adenomatous polyp to colorectal carcinoma or metaplastic BE to dysplastic BE to EA), with specific genomic alterations being required to drive phenotypic progression to the next stage (Fig. 11.1). This view of cancer evolution suggests a certain predictability in tumor development, where particular alterations are required for progression at a given point in the process, and the underlying concept that determining risk of progression can be accomplished by identifying those specific genomic alterations known to be associated with more advanced stages. As well, it suggests that interruption of the sequence of progression by targeting the gene or genes involved will stop future progression to cancer—but which genes are involved in which cancers, and how do they function to drive progression?

The first drafts of the human genome were published in 2001 [7], providing a roadmap for the ongoing efforts to understand all the somatic genetic and epigenetic alterations in the genome that are responsible for the development of cancer [8]. Through the efforts of programs like The Cancer Genome Atlas and the International Cancer Gene Consortium, more than 30 types/subtypes of cancer have undergone whole genome and/or whole exome sequencing to characterize the vast array of small- and large-scale genomic alterations that can occur during the development of cancer. In general, these studies have consisted of single samples taken from a cancer at a single point in time, generating a snapshot of the genomic alterations present at that particular location in the tumor at that particular moment. These studies have successfully produced lists of genes frequently altered in end-stage cancers, potentially identifying actionable therapeutic targets. Unfortunately, the hoped-for identification of genes that are altered at a high frequency within a cancer type, which would allow the steps in the linear pathway discussed earlier to be filled in, has not occurred. In sporadic cancers, with the exception of *TP53*, few genes are altered in more than 10 % of cancer samples, with a large number of genes being altered in small minorities of cases [9]. Rather than identifying the smoking gun(s) responsible for "driving" the development of a particular cancer type, these studies have demonstrated the heterogeneity that can exist at the level of gene mutations across cancers in the population, and that there are likely many paths to a malignancy. In addition, the characterization of *intratumor* heterogeneity in diverse cancers from the kidney [10], lung [11, 12], colon [13], breast [14], and esophagus [15] underscore that not only are evolutionary processes occurring prior to the development of cancer, but these processes don't stop once a neoplasm achieves the histologic definition of cancer.

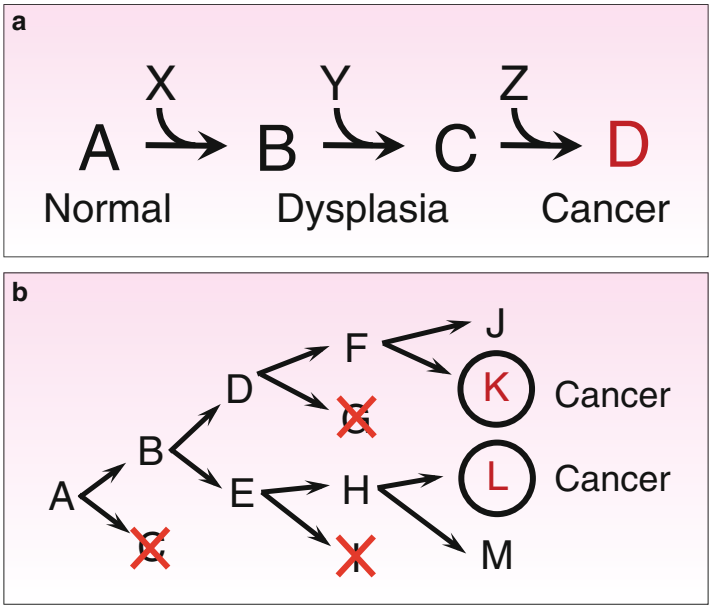


Fig. 11.1 Linear and branched models of cancer development. (**a**) The linear model of cancer development as has been taught in medical school for nearly 100 years. Development proceeds in a predictable fashion, with genetic alterations at each step being required to generate the phenotypic changes associated with the traditional histology-based assessment of progression (e.g., normal to dysplasia to cancer). Identification of early stage alterations (e.g., mutation in gene *X*) indicates a patient at risk for developing cancer, while later stage alterations (e.g., alterations in gene *Z*) can be used for early cancer detection. (**b**) The branched evolution model of cancer development was integrated and presented by Nowell in 1976. Progression proceeds through selection of variants having survival/growth advantages. Some lineages (e.g., C and G) are outcompeted by those with better fitness, while other lineages develop additional alterations that eventually lead to cancer (A → B → E → H → L). At the population level, there are a large number of pathways that can lead to a cancer diagnosis and subsequently a large number of genes found altered in cancer samples

The results of the projects to analyze cancers at the levels of the genome, epigenome, transcriptome, and proteome have provided a wealth of information that have aided our understanding of the biology of cancer. At the same time, the basic questions we hoped would be answered still remain; what are the genetic alterations required for a given cancer and how do they develop and evolve over time? These results emphasize a perhaps unexpected result of sequencing cancer genomes. Rather than identifying all the alterations that are required for the development of a cancer, these studies instead identified all the ways the genome of a cancer—or, more accurately, the genomes of a population of cells from one location from within a cancer at one particular point in time—can become disrupted via a large number of intrinsic and extrinsic processes, both selected and unselected. Whereas some of these alterations reflect those required for the development of the cancer (often referred to as "drivers"), many are consequences of the ongoing

genomic instability in the neoplastic cells ("passengers"), although some of the alterations may become important at later time points. Thus, most of the alterations detected through these sequencing efforts don't necessarily provide information about how to detect a particular cancer early or how to determine a person's risk of developing cancer (Fig. 11.1), although they may provide information about specific genetic alterations that may become targets for later stage therapies. Accomplishing the critical goals of early detection and risk prediction requires examining multiple individuals in the population and comparing the types of alterations that develop in those that do progress to cancer versus those that do not [16].

A specific example, esophageal adenocarcinoma, illustrates how the complexity of neoplastic progression has impacted the conclusions that can be drawn from cancer sequencing studies. Genomic alterations have been characterized in EA by single nucleotide polymorphism (SNP) arrays and more recently by either whole exome or whole genome sequencing [15, 17–21]; a more complete catalog of EA alterations will be available with the publication of the TCGA analysis. These studies reported that the mutational load in EAs is high (median of 16,994 [21] to 26,161 mutations/genome [18]), exceeded only by melanoma, bladder and lung cancer, and that microsatellite instability was rare (~4 % of EAs) [18]. These alterations appear to be arising through a number of mutational processes [15, 19, 22], including a higher than expected frequency of AA to AC transversions at AA dinucleotides that has only been observed in adenocarcinomas of the esophagus and stomach [23–25]. Consistent with the observed high mutation rate, EAs were characterized by a large number of genes containing point mutations/small indels. Dulak et al found 8331 genes that were mutated in at least one of the 149 EAs they sequenced, with a median of 106 nonsilent mutations per non-MSI EA [18]. However, despite this heavy mutational load, very few genes were altered in more than 20 % of the cancers, similar to the findings from the other studies. Only *TP53* was altered in a majority of the cancers [17–19, 21], but the ubiquity of mutations across nearly all solid tumors indicates *TP53* is not a unique driver of EA. The extensive copy number alterations and structural rearrangements found in each cancer indicate a variety of mutational mechanisms are active during progression to EA [18]. The data from these studies indicate EAs develop in a highly mutagenic environment (gastroduodenal reflux, inflammation) that leads to a large number of mutations and copy number alterations, but very few of these genomic changes occur in more than 10 % of the cancers. Importantly, the small number of studies that examined the BE tissue around the EA found clonally related alterations in the precursor and the cancer [17, 20], indicating the evolution of the somatic genomic alterations found in EA begins in BE. As well, there is heterogeneity within each EA, demonstrating that evolutionary processes do not stop once a cancer is diagnosed [15]. Perhaps presciently Nowell stated almost 40 years ago "It is not surprising, therefore, that consistent alterations from case to case, either antigenic or metabolic, have been difficult to identify in the common human solid malignancies" [1].

Barrett's Esophagus and Esophageal Adenocarcinoma: A Model System for Studying the Development and Progression of Cancer In Vivo

Disruption of gene function is a central tenet for the development of cancer and cataloguing the genomic alterations in human cancers that affect normal gene function is a critical ongoing process. However, an equally critical need is an understanding of how the cell populations with these alterations develop and evolve over time and space in the patient. Here we present a model system for evaluating how a solid tumor can develop and evolve over time and space in vivo. BE is a condition in which the normal stratified squamous epithelium lining the esophagus is replaced with a metaplastic, intestinalized epithelium as a result of chronic gastroesophageal reflux [26–28]. Patients with BE are at a higher risk of developing EA, a cancer that has increased dramatically in incidence over the past 30 years and has a poor 5-year survival rate unless detected early [29]. BE is viewed as both a precursor of cancer and a benign adaptation to the harsh environment of the reflux exposed esophagus. Barrett's epithelium much more effectively resists the damaging effects of chronic reflux than squamous epithelium, due to increased expression of mucosal repair and defense genes [30], secretion of a thick layer of mucus containing bicarbonate ions [31, 32], and claudin-18 containing tight junctions [33]. Why some patients with chronic reflux develop BE whereas others do not is not well understood; a discussion of this question and the related topic of the origin of the Barrett's epithelium is beyond the scope of this review, but is a question that has implications for prevention and therapy [34–37].

Because of the poor survival associated with late stage EA, the standard of care for patients with BE has been periodic endoscopic surveillance for the early detection of cancer [28]. However, the natural history of BE and EA presents a clinical dilemma: more than 90 % of patients diagnosed with BE will not progress to EA in their lifetimes, while more than 90 % of the cases of EA are diagnosed in individuals who were not previously diagnosed with BE (i.e., not currently in surveillance [38]). Progression to EA in BE patients is lower than previously thought, with recent population-based studies estimating a 0.12–0.43 % risk of progression per year [39–41]. In this respect, BE is similar to other premalignant conditions, such as those in the breast and prostate, in which accurate determination of progression risk are difficult, leading to significant overdiagnosis of non life-threatening disease and overtreatment of patients who will not benefit and instead may be harmed due to invasive testing and unnecessary treatment [42]. In BE patients, as in those with other premalignant conditions, a better understanding of the natural history of how cell populations with genomic alterations evolve in both progressors and nonprogressors is necessary to more accurately assess risk of progression and to target interventions to those who will benefit from them.

The standard of care for BE patients of periodic endoscopic surveillance for the early detection of cancer allows biopsies to be taken spatially throughout the BE segment and sequentially at multiple time points, making possible characterization of

how the genomes of cell populations evolve over space and time before the development of EA. This ability to safely obtain multiple BE biopsies at successive endoscopies [43] is nearly unique; most premalignant tissues are not detected unless they cause symptoms (e.g., prostate) or are detected through random screening (e.g., during mammography or colonoscopy), and when a tissue is found that is considered to have malignant potential, the standard of care is removal of the tissue and a wide margin around it to reduce risk of progression, making later analysis of the evolution of genomic alterations in the excised tissue impossible. Since removal of the esophagus is not a reasonable preventive treatment, BE is an excellent system for characterizing how cell populations with genetic alterations develop over time and space.

The Development of Evolution Studies in BE and EA

Although the tools and techniques for accurately detecting and characterizing somatic genomic alterations, such as high density SNP arrays, next-generation sequencing and the bioinformatics platforms required for analysis, have only been in common use over the past decade, earlier studies have provided valuable information on characteristics of neoplastic evolution in BE and EA. The discussion later focuses primarily on studies from our laboratory and shows how these types of analyses progressed from earlier cancer-only studies to more complicated study designs; for those interested, more comprehensive reviews of genetic studies in BE are available [27, 44]. An early observation of genomic alterations in EAs was that of frequent aneuploidy, a finding that has been replicated in many laboratories and in more recent pan-cancer analyses [45]. The study by Rabinovitch et al. in 1988 [46] examined multiple samples from unfixed esophagectomy specimens, finding multiple aneuploid populations (as characterized by DNA content flow cytometry) in and around the EA (Fig. 11.2). Measures of ploidy by flow cytometry are sensitive in that the DNA content in each individual nucleus in a sample is measured, giving an accurate representation of the clonal populations in a mixture, with the ability to isolate cells having measurably different ploidies for other analyses; however, flow cytometry is limited in resolution, with differences in ploidy between two samples of less than 10 % being difficult to resolve reliably. As well, two clonal populations may have very different genomic alterations that independently result in the same ploidy, making them impossible to distinguish. Despite these limitations, early studies of advanced EAs showed the presence of a mosaic of clonal populations with distinct ploidies across space in the surgical resections of these cancer patients, indicating that at some point during the development of the EA, multiple clones had developed and expanded across the Barrett's epithelium.

The 1999 study described in Barrett et al. characterized alterations in genes/chromosomal regions suspected to play a role in progression, as well as development of tetraploidy and aneuploidy, in BE patients who progressed to EA while in surveillance [47]. This study expanded upon earlier work by examining multiple samples over both time (from multiple endoscopies) and space (multiple samples

were examined per endoscopy and/or from surgical resections). The results from these analyses demonstrated that while the development of EA was characterized by recurring somatic genomic alterations (development of aneuploidy/tetraploidy, mutations in *TP53 and CDKN2A*, LOH at tumor suppressor loci), there were different pathways to the development of each individual cancer (Fig. 11.2). The development of EA did not follow a common linear pathway for all patients; instead, the specific environmental pressures present in a given individual's esophageal tissue yielded a unique pathway to cancer in each neoplasm. This study also used a limited type of phylogenetic analysis, clonal ordering [48, 49], to infer which alterations tended to occur earlier during the process of progression than others. However, this study examined only a limited number of preselected genomic alterations, instead of a nonbiased genome wide evaluation, and only characterized patients who progressed to high-grade dysplasia (HGD) or EA.

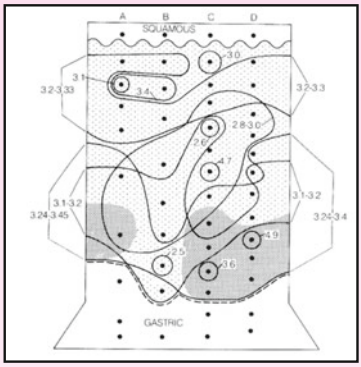
In 2006, a study by Maley et al. utilized measures of clonal diversity originally developed for studies in ecology and evolution to examine risk of progression to EA in BE patients [50]. This prospective study expanded upon previous work by evaluating cell populations in patients who did not progress to cancer in addition to those that did progress to EA, and focused upon whether the extent of clonal diversity present in the BE segment was predictive of risk for subsequent progression to EA in a cohort of 268 patients followed for an average of 4.4 years. Clonal diversity was assessed using LOH and microsatellite size shifts at 19 loci throughout the genome, sequence mutations in *TP53* and *CDKN2a*, and changes in DNA content by flow cytometry. Significant associations between increased measures of clonal diversity (number of clones, genetic divergence or Shannon index) and progression to EA were found (Fig. 11.2), suggesting that generating diverse cell populations may be a "fundamental evolutionary mechanism of neoplastic progression with profound clinical implications" [50].

The data collected in the three previous studies informed the analysis presented by Galipeau et al. in 2007 that further demonstrated the utility of using these genomic alterations for overall risk prediction for progression to EA [51]. This prospective study found a panel of three biomarkers (LOH on chromosomes 9p and 17p and DNA content aneuploidy/tetraploidy) was effective in identifying patients at risk for future development of EA; patients with alterations at all three biomarkers had a dramatically increased relative risk of progressing to EA of 38.7 (95 % CI 10.8–138.5, $p < 0.001$). Importantly, this study also found consistent use of nonsteroid anti-inflammatory drugs (NSAIDs) such as aspirin or ibuprofen was associated with reduced risk of progression to EA, even in those patients having alterations in all three biomarkers. These data suggested that chemopreventive agents are capable of modulating the selective conditions and evolutionary path of cell populations in the reflux-exposed esophagus. If cancer is indeed a disease of the genome and NSAIDs work to reduce the risk of developing cancer, it should be possible to determine how NSAIDs are altering the evolutionary processes that lead to cancer by measuring their effects at the level of the genome (e.g., the development and evolution of mutations and copy number alterations) [52].

a

Finding: The esophagus of EA patients is a mosaic of genetically diverse cell populations

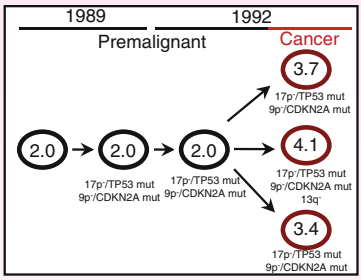
Key study element: Analysis of multiple samples over space



b

Finding: EA can develop through multiple pathways

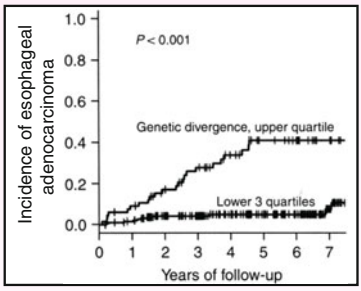
Key study element: Analysis of samples over both space and time in multiple patients



c

Finding: Genetic diversity measures identify risk of progression to EA

Key study element: Integration of diversity measures from ecology and evolution



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Finding: NSAIDs reduce risk of progression in low and high risk patients

Key study element: Changing the environment can modulate neoplastic evolution

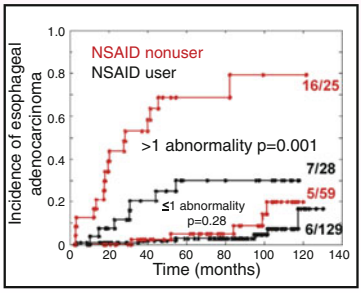

Fig. 11.2 Evolution studies in BE and EA. The study of how cancer evolves in vivo requires analysis of multiple samples over both space and time in the developing neoplasm. The standard of care for patients with BE, periodic endoscopic surveillance with biopsy for early detection of EA, allows these samples to be obtained in a safe manner [43, 84]. Studies performed over the past

Necessary Elements for a Study of Neoplastic Evolution

The sequential selection of neoplastic cell populations requires a selective environment that favors cell populations that have undergone genetic alterations that increase their relative survival [1, 53]. The harsh environment of the esophagus that is chronically exposed to gastroduodenal reflux certainly selects for the establishment of the Barrett's epithelium and contributes to development of EA. Population-based studies consistently find an association between the severity and duration of reflux symptoms and the risk of progression to cancer [54–56], and progression models find harsh selective environments lead to development of aggressive, invasive tumors [57]. This environment also leads to the generation of variants having genetic alterations, through direct mutagenesis from components of refluxate [58] and indirectly through acute and chronic local and systemic inflammation [59, 60]. The tissue damage done to the esophageal epithelium by means of the chronic reflux results in a substrate for the growth of genetically variant cell populations that continue to evolve across the surface of the esophagus over space and time.

An ideal study characterizing the evolution of neoplastic cell populations in vivo requires sampling over space and time at sufficient density to detect clonal populations and to follow them as they either progress to cancer or remain a stable, benign neoplasm. While this is a straightforward concept, practical considerations make this sampling strategy difficult in reality. As discussed earlier, the most common obstacle is the accessibility to sample tissues at spatially distinct locations across multiple time points. Thus, many studies of neoplastic evolution

Fig. 11.2 (continued) 30+ years have characterized the types of genomic alterations that develop in patients with BE and EA. The examples described here demonstrate various study designs that have been used to understand how cell populations with these alterations evolve over space and time. (a) Rabinovitch et al. [46] described the presence of overlapping cell populations with aneuploid DNA content in surgical resections from patients with advanced EA, demonstrating that multiple cell lineages can develop in a single patient. Each black dot indicates a biopsy location in the surgical resection; lines delineate areas with similar aneuploid cell populations as determined by flow cytometry; dark shading indicates location of cancer, light shading is the Barrett's segment. (b) Barrett et al. [47] used multiple samples across multiple time points to characterize how EA can evolve along a unique pathway in each individual. Numbers in the circles indicate ploidy as determined by flow cytometry, data under circles indicate presence of LOH on chromosomes 9p and 17p and mutation status of CDKN2A and TP53. (c) Maley et al. [50] described the use of measures of diversity, adopted from studies of ecology and evolution, to show that the presence of genetically diverse cell populations in the esophagus of BE patients was associated with increased risk of progression to EA. Graph indicates the difference in progression to EA in patients with the highest quartile of genetic divergence between multiple samples taken at baseline endoscopy in a cohort of 268 BE patients with 37 EA outcomes. (d) Galipeau et al. [51] demonstrated how NSAIDs modulate the evolution of cell populations in BE patients and reduce the risk of progression to EA. Patients were grouped according to their number of risk abnormalities (baseline 9p LOH, 17p LOH, and/or DNA content tetraploidy/aneuploidy) and stratified into current NSAIDs users or nonusers. In both high risk (>1 risk abnormality) and low risk (≤1 risk abnormality), NSAID users had significantly lower risk of progressing to EA

have focused upon already diagnosed cancers, allowing cataloguing of alterations found in a cancer type, but not discrimination of what alterations are specific to patients who progress to cancer versus those who don't progress. Neither does it indicate the types of alterations that occur before the diagnosis of cancer, which are critical for development of early detection and risk prediction strategies. BE provides a rare opportunity to investigate a much broader spectrum of neoplastic evolution in vivo, with samples being obtained from both patients who do and do not progress to cancer. However, even when using BE as a model system, additional considerations complicate the development of designs to study somatic genomic evolution. The difficulties in maintaining an active cohort of patients over the years to decades required to collect, process, and store prospectively obtained biopsies and to accumulate enough sufficient cancer endpoints to power a study are difficult to overcome, especially in the current era of reduced funding for long-term clinical studies. As technologies advance that allow formalin fixed paraffin embedded (FFPE) material to be accurately genotyped and sequenced [61], it is likely that biorepositories of well annotated samples that were used for histologic examination may be utilized in genetic studies that incorporate the design elements discussed here.

Temporal and Spatial Evolution of Somatic Chromosomal Alterations

We will examine in more detail the study by Li et al. [16], which describes what is to date the largest analysis of the evolution of neoplastic cell populations in vivo with a cancer outcome. The following sections outline the critical aspects of the study design and the results, and discuss their implications on how BE can evolve over time and space to either a benign stable condition or to a cancer endpoint (Fig. 11.3).

Fig. 11.3 (continued) to last endoscopy (progressors) or last endoscopy (nonprogressors) during which biopsies were taken. (b) Isolation of Barrett's epithelium removes stromal cell contamination that would reduce sensitivity for detecting SCA. The crypt structure of the Barrett's epithelium and the honeycomb appearance of the remaining stroma are clear in this image. (c) SNP arrays allow an unbiased analysis of copy number changes and LOH throughout the genome. Whole genome sequencing would provide this information at increased resolution, but at a significantly higher cost per sample. Image shows copy number alterations throughout the genome, indicative of a genome doubling event, in a sample from a patient who progressed to EA. (d) Multiple samples were analyzed at each of two time points to characterize cell populations and how they evolved over time. Image indicates biopsy locations and amount of SCA present in each biopsy from a progressing patient taken at two time points. Colors indicate relative contribution of different alteration types (blue = copy neutral LOH, green = copy loss, orange = copy gain, *black* = homozygous deletion). Size of circle approximates amount of SCA in megabases (Mb). Adapted from [16]

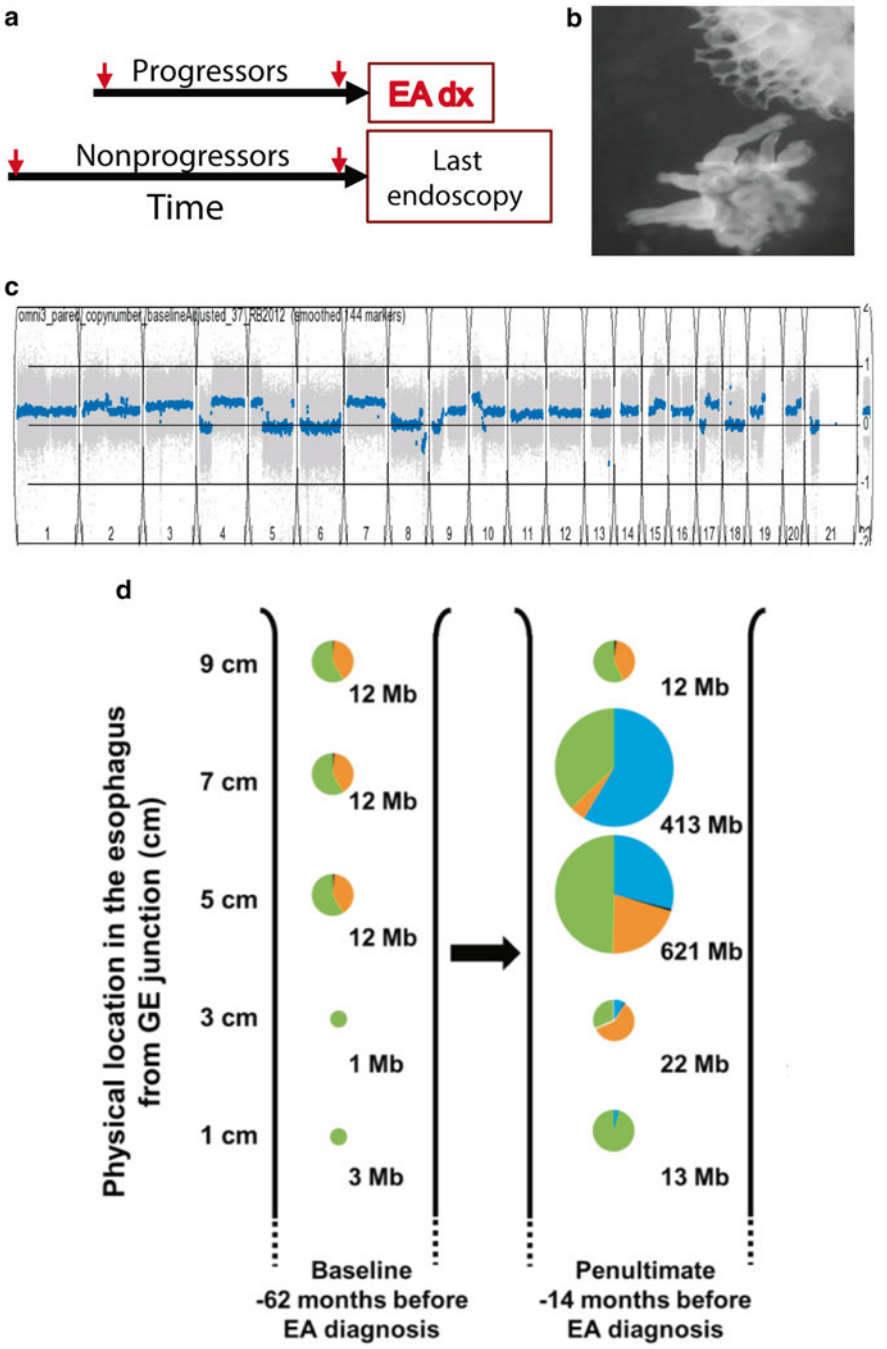

Fig. 11.3 Elements of study design for assessing temporal and spatial evolution in BE. (**a**) Comparing the genomic alterations in patients who progress to an EA endpoint with those who do not allows discrimination of the alterations required for progression. Vertical arrows indicate baseline and next

Study Type

A case cohort study [62], which captures all of the cases in a cohort as well as randomly selected nonprogressing cases for comparison was used since it allows identification of the types of alterations specific to progression in addition to those found in nonprogressors. This identification is especially relevant for patients with BE since the vast majority of them (90 %) will never progress to EA. This approach is economical (all cases are examined) and the random selection of the nonprogressors preserves the integrity of the cohort for later analyses. A critical aspect of this approach is that the control population selected for this study was validated, nonprogressing BE patients, not unaffected individuals, allowing determination of the genomic changes that characterize indolent, nonprogressing BE. Because of this study design, it is more accurate to state this was a *comparative* study of spatial and temporal evolution between BE progressors and nonprogressors, a relevant point considering that these are the two patient populations that need to be discriminated at the clinical level.

Epithelial Isolation

Many studies use either a cutoff (often 70 %) for the percentage of cells of interest (e.g., cancer cells) in a sample or increase the percentage of cells of interest using techniques such as laser capture microdissection to reduce background signals from the surrounding normal stromal cells. We used an epithelial isolation technique originally developed for isolating colonic crypts [63, 64] to physically separate the Barrett's epithelium from the underlying stroma, (Fig. 11.3) yielding a >97 % pure Barrett's epithelium.

Use of SNP Arrays to Identify Somatic Chromosomal Alterations

At first glance, whole genome sequencing appears to be a better approach for this study, since it allows point mutations, indels, structural rearrangements, and copy number alterations to be assessed simultaneously. However, at the time this study was conducted (and even now), this benefit comes at a significantly higher cost per sample than SNP arrays. Thus, there is a tradeoff between the increased resolution at the level of the genome provided by whole genome sequencing versus the increased spatial and temporal resolution provided by analyzing a greater number of samples using SNP arrays. Copy number alterations, LOH, and changes in ploidy have been shown to be common events in the development of EA [27, 65, 66], suggesting these types of alterations are selected events during the development of EA. Given that the goal of the study—comparison of the spatial and temporal evolution of chromosomal alterations in progressors

and nonprogressors—involved analysis of 1272 samples, we chose SNP arrays, with the caveat that we would not be able to determine if specific point mutations or structural rearrangements were associated with progression to EA.

Evaluations of Samples Over Time and Space

The evolution of cell populations with genomic alterations over space was assessed by examining *randomly* selected biopsies from every two cm of each patient's BE segment (average of 2.4 and 3.1 biopsies per patient per time point in nonprogressors and progressors, respectively). The evolution over time was assessed by examining biopsies from two endoscopies performed at two time points: one at the initial baseline endoscopy and the other at either the next to last or last endoscopy available for the patient. All of the alterations identified in the samples were compared to a constitutive normal sample, derived from either blood or normal gastric tissue obtained during endoscopy.

Cancer Endpoint

The endpoint for this study was progression to EA. HGD is frequently used as a surrogate endpoint for EA, but it does not meet the requirements for a valid surrogate endpoint [67, 68] since it is poorly reproducible [69–71] and it has a highly variable association with progression to EA [72–75]. Diagnoses of EA are very reproducible and allow the results of this study to be compared to those of any other study using EA as an endpoint. Use of HGD as an endpoint introduces a level of variability that makes cross study comparisons impossible. Of note, very few of the patients in this study had undergone any type of therapy (four progressors and 3 nonprogressors (total 2.8 %) had undergone EMR at some point between baseline and the last time point), so the evolution of chromosome alterations we observed reflected the natural history of the BE in the vast majority of the cases. The increasing frequency at which ablation and EMR are being used in the general BE population is likely to make this type of study of the natural history of BE progression more difficult, if not impossible, in the future.

The study described here was designed to provide a comparison of the natural histories of cell populations in patients with benign versus progressing BE over a median of more than 64 months of follow-up time. The characteristics of the somatic chromosomal alterations (SCA, which are copy gain, copy loss, homozygous deletion, and copy neutral LOH) which developed and evolved over time and space in the nonprogressing population were significantly different than those of the progressors.

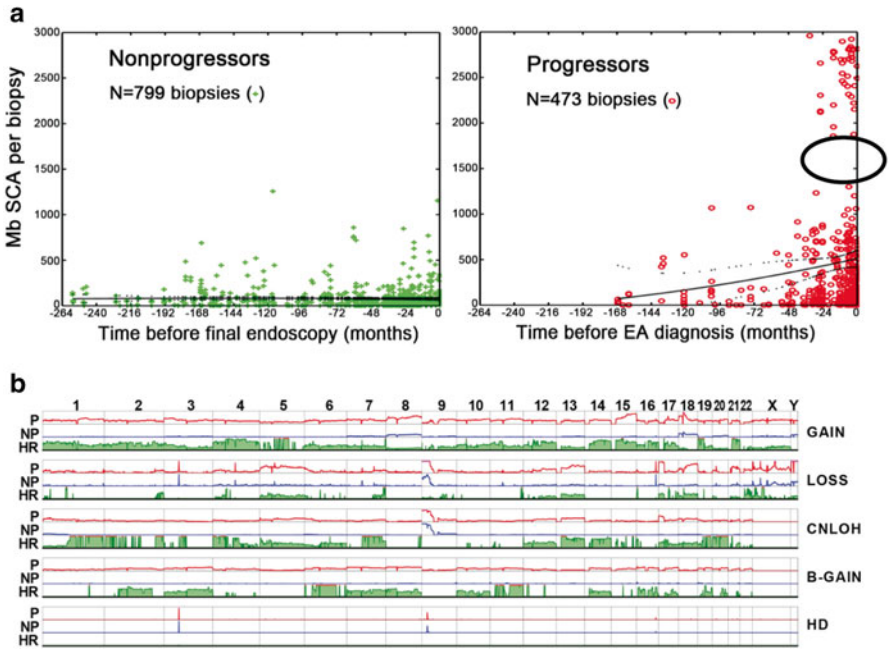

Fig. 11.4 Evolution of temporal and spatial evolution in BE. (**a**) Comparison of the levels of SCA that develop over time in populations of patients who do or do not progress to EA. *X*-axis represents time before last endoscopy or before EA diagnosis in nonprogressors and progressors, respectively, *Y*-axis represents total SCA in mbp per biopsy. Solid black lines indicate trend in mean SCA (*dotted black lines* 95 % CI of the mean). Black circle indicates gap between 1300 and 1850 mbp SCA. (**b**) Comparison of frequency of alterations in progressors and nonprogressors identify genomic changes that can discriminate risk of progression. Red line indicates frequency of alterations in progressors (P), *blue line* indicates frequency of alterations in nonprogressors (NP), and *green line* indicates hazard ratio (HR) for progression to EA for given alteration. High-frequency alterations in both progressors and nonprogressors have low hazard ratios; alterations that occur at moderate to high frequency in progressors but low frequency in nonprogressors are associated with higher hazard ratios. Adapted from [16]

Nonprogressors

As a population, BE patients who did not progress to EA showed consistently low levels of SCA from their baseline evaluations up to their most recent endoscopies (Fig. 11.4), with 95 % of the biopsies from these patients having less than 283 mega-basepair (mbp) SCA. At the level of SCA as assessed by SNP arrays, the benign BE displayed by these patients is characterized by stable genomes both throughout the esophagus and over time. The nonprogressors in this study represent the 90–95 % of BE patients in surveillance who do not progress to EA in their lifetimes and indicate the successful adaptation of Barrett's epithelium to the damaging environment of the reflux-exposed esophagus [76]. However, nearly all of the nonprogressing BE patients still exhibited measurable levels of SCA in the Barrett's epithelium.

Whether these alterations are necessary for the development or spread of the Barrett's epithelium is not currently known. Even though some nonprogressors had multiple clonal populations present in their BE segments, these populations were significantly less genetically diverse over both time and space when compared to the progressors. Thus, the nonprogressing patients showed reduced generation and spread of cell populations with genetic variants, two of the critical components for neoplastic evolution.

Importantly, some of the alterations found in the nonprogressors included SCA previously identified as high-frequency changes in EA, including alterations (double deletions, single copy loss, and/or copy neutral LOH) at chromosome fragile sites *FHIT* and *WWOX* and in and around *CDKN2A* [77–79] (Fig. 11.4). The pervasiveness of these alterations suggests these genomic locations may be inherently prone to breakage/damage under the selective conditions of the reflux-exposed esophagus. Alternatively, changes in the functions of the proteins encoded by these genes themselves may be selected for and play a role in the initial development of the Barrett's segment [80]. This finding demonstrates the value of a comparative study of evolution, and why evaluating the frequency of alterations in cancers *in vacuo* may be insufficient for identifying biomarkers for early cancer detection or cancer risk prediction.

Progressors

The characteristics of the SCA in the progressor population directly contrast those found in the nonprogressors. As a population, the genomes of patients who progressed to EA showed a significant *increase* in the amount of SCA beginning about 4 years prior to the diagnosis of cancer (Fig. 11.4). As well, there were significantly higher levels of genetic diversity between cell populations over both time and space in the esophagi of patients who progressed to cancer, also in the 4 years prior to the diagnosis of EA. Thus, progressing patients show both increased generation and spread of cell populations with genetic variants. These data support previous studies showing that diversity measures from ecology and evolution are valuable adjuncts for identifying patients at risk for progressing to cancer [50].

There was a dramatic increase in SCA in a subset of patients consistent with genome doubling events (development of aneuploidy/tetraploidy). The absence of samples with SCA levels between 1200 and 1800 mbp (Fig. 11.4a) indicated this was not a gradual increase in the amount of the genome undergoing alterations but was likely the result of discrete events affecting most or all of the genome. Such catastrophic, genome changing events are being increasingly reported in a wide variety of cancers, indicating that punctuated evolution is a characteristic of many cancer types. These data support pan cancer analyses showing that genome doublings are common in EAs [45] and are consistent with the later study by Nones et al. showing chromothripsis [81] occurring in 36 % of EAs [19].

Comparison of the frequency of different types of alterations in nonprogressors and progressors allows alterations that are associated with progression to EA to be

distinguished from those that simply characterize the development of BE. Figure 11.4b illustrates the frequency at which different types of alterations were found throughout the genome at all times before a diagnosis of cancer (progressors) or the last endoscopy (nonprogressors). Some regions were found to be altered at high frequency in progressors (e.g., loss on chromosome 3p or loss/cnLOH on chromosome 9p), but these alterations occurred at high frequency in nonprogressors as well, meaning they are not good discriminators of progression risk. Other alterations that occur at intermediate frequencies in progressors (e.g., regions of cnLOH on chromosome 5, 6, and 7, large regions of balanced gain throughout the genome that are associated with genome doubling events) are very rare in nonprogressors and are associated with higher hazard ratios for progression to EA. Taken together, these data can be used to identify a panel of alterations that can be employed to determine a patients risk of progression [82].

In contrast to point mutations, the mutational events that generated the SCA in the progressors affected large portions of the genome, with alterations in multiple regions of the genome being coselected (Li et al. Supplementary data Fig. 11.4). Large-scale alterations such as gain or loss of whole chromosomes affect the expression of many genes simultaneously, rapidly generating genetic heterogeneity. This heterogeneity may explain why alterations in specific genes are not found in a majority of cancers, with dysregulaion of common pathways more frequently observed [9, 83].

While sampling over two time points was not sufficient to comprehensively track the developmental history of all the cell populations in each progressor, analysis across the entire cohort revealed a significant increase in the level of SCA in the progressors 4 years prior to a diagnosis of EA. Thus, at the level of measuring total SCA (total bp of the genome affected by SCA) by SNP array, there is a 4-year window in which a potential progressor might be identified in a population of BE patients. Translation of these findings to a clinically relevant platform that could rapidly and accurately assess a patient's risk of progression in the near future (i.e., <4 years), perhaps using material from FFPE biopsies obtained for histologic assessment, are ongoing.

Other Considerations

No study can answer all questions, and the vagaries of clinical research require understanding where interpretation of the data can be confounded by uncontrollable factors. Even though the biopsy protocol for patients in the Seattle Barrett's Esophagus Cohort is high density (targeted biopsies of suspicious lesions, plus 4-quadrant biopsies every 1–2 cm in the BE segment) [84], the number of samples analyzed for *genetic* alterations for each patient in this study (1 every 2 cm of the BE segment) only covered a small portion (~2%) of the available surface area of the Barrett's segment. The primary goal for the patients in this cohort was early detection of cancer; subsequently, many more (~10×) biopsies were taken at each endoscopy/time point for histologic evaluation compared to those taken for genetic analyses. Thus, it is likely that some clones with chromosomal

alterations were missed in this study. Increasing sampling density over time and space would alleviate this issue, with the tradeoff of increased cost. The development of techniques to representatively sample the entire BE segment, such as the Cytosponge [85, 86] may allow better detection of specific alterations in a clinical setting.

The use of arrays in this study allowed comparison of how copy number alterations and copy neutral LOH develop and evolve in BE patients who do and do not progress to EA and identified a 4-year window of opportunity in which patients who will progress to EA may be identified. It is possible that this window may be extended either by more precise selection of markers of instability (e.g., identifying a specific panel of alterations that has better sensitivity/specificity for EA than overall SCA level) or by evaluating other types of genome changes (point mutations, structural genomic rearrangements, or epigenetic alterations) through whole exome or whole genome sequencing.

The genetic analyses performed in this study (and in most other genomic studies) used bulk biopsies, meaning that the genomic changes detected were averages of all the cells in the biopsy. We estimate an average BE jumbo biopsy contains between 200 and 600 crypts (data not shown). Alterations in cell populations that made up a small minority of the biopsy would not necessarily be detected in the bulk readout, even though they may contain clones that may later progress to cancer. Work done with single-cell sequencing has uncovered unsuspected heterogeneity in breast cancer [24, 25], and sequencing of individual crypts from patients with colon cancer [13] has characterized how clonal populations spread through space and time. This level of characterization would not have been possible if the analyses had been based upon data from bulk biopsies.

Finally, the observer effect, in which the process of making a measurement within a system causes a change in that system [87, 88], has to be considered. During endoscopic surveillance, the process of taking a biopsy removes cells from the body that will no longer evolve. Although many of the cell populations we characterized were found in multiple biopsies, indicating they had spread across the surface area of the Barrett's segment to a size much larger than a single biopsy (see Figs. 11.2a and 11.3c), the cells that were characterized in the study are no longer involved in the evolution of cell lineages in the patient. This change particularly has implication for phylogenetic analyses in which cell populations are followed over long periods of time to characterize their evolution [52].

Questions and Implications for Future Studies of Cancer Evolution

"The stepwise sequence in each tumor differs (being partially determined by environmental pressures on selection), and results in a different, aneuploid karyotype in each fully developed malignancy" [1].

Nowell made this conclusion in his study of the clonal evolution of neoplasia in 1976; the same sentence could describe the overall results of cancer sequencing across multiple tumor types that have occurred over the past decade. Whereas our understanding of which types of genetic alterations occur in cancer has progressed dramatically, our understanding of how these alterations originate and evolve over time and space are still developing. Analyses of cancer genomes in general and more specific studies in BE and EA patients have suggested some answers as to how neoplasms evolve in vivo.

The linear model of cancer development, in which malignancies develop along a predictable pathway, makes certain predictions about how cancers can be detected early or risk of progression assessed, as well as how malignancies can be treated or prevented. The deterministic linear model suggests that risk assessment and early detection of cancer require identifying specific diagnostic alterations; if an early stage alteration is detected, then the patient is at risk of progression, while a later stage alteration would indicate a cancer may have already developed (Fig. 11.1). Prevention would target those genes that are altered early in the pathway, breaking the "chain of progression" to cancer by preventing the later stages from occurring. The branched model makes very different predictions. Having multiple pathways to cancer means early detection would at the very least require a panel of markers that could cover the range of possible genetic changes involved across the multiple progression branches. Developing such a panel may involve identifying characteristics that indicate the presence of rapidly evolving cell populations, such as aneuploidy or high levels of copy neutral LOH. Prevention would require targeting the process of evolution itself; that is, slowing the process down by either altering the selective environmental pressures or reducing the rate at which genetic variants are being generated. A branched evolutionary model means treatment of cancers would be more difficult, since the number of common, targetable genetic alterations in each cancer type would be small, with each tumor capable of generating new variants that were resistant to a given treatment.

The wealth of data being generated through sequencing of cancer samples is not consistent with the linear model of cancer development. Common genetic alterations are not being identified within cancer types [9]; instead, substantial genetic heterogeneity exists not only between tumors of the same type, but within tumors as well [11, 13, 15]. Focusing on studies in the esophagus, while individual EAs have some characteristics in common (high levels of SCA, frequent aneuploidy, involvement of p53 alterations) there are clearly multiple pathways through which a cancer can develop [15, 18, 47]. A linear model of carcinogenesis predicts that the development of clonal cell populations with new alterations that provide a growth advantage will lead to expansion of those clones, reducing overall diversity in the growing neoplasm. Li et al found the opposite, that heterogeneity remained high in individuals who progressed to cancer compared to those who maintained stable, benign disease [16], consistent with findings reported recently by Sottoriva et al. in the development of colorectal cancer [13] and earlier studies showing increased diversity predicts progression to EA [50]. Interventions proven to be successful in reducing risk of progression to EA, such as NSAIDs [51, 89], have been those that

target overall mutational processes, as opposed to targeting single genes or pathways. It is also apparent that cell populations continue to evolve after the cancer is diagnosed [11, 13, 15]. All of these data are consistent with cancer developing through the branched evolutionary pathway outlined by Nowell.

The rate at which the genomes of cell populations become altered over time is difficult to measure directly, requiring sampling clonally related cell lineages over multiple time points. Certainly some mutational processes occur at a slow, gradual rate, such as those generated through the mutational signature associated with aging [22] or the erosion of telomeres over multiple cell divisions [90]. However, growing evidence indicates that the development of genomic alterations and the evolution of cancer can occur in rapid, punctuated bursts. Whole genome doublings, which can lead to aneuploidy [45], chromothripsis [81], and chromoplexy [91] are events that can be generated in a single or small number of cell divisions, affecting large portions of the genome and hundreds to thousands of genes simultaneously, and have been found in EA samples from multiple studies [18, 19, 45]. In Li et al. [16], there is a nonlinear increase in the amount of SCA starting 48 months prior to a diagnosis of cancer (Fig. 11.4a), and the lack of samples with levels of SCA between 1300 and 1850 mbp indicates the development of aneuploid populations did not occur through a slow gradual process with intermediate levels of alterations, but were likely generated through a process of genome doubling. Such a distinction between gradualism and catastrophic, punctuated genomic changes is more than just academic, for the rate at which cancers develop impacts our abilities to detect and treat them. Rapidly evolving neoplasms will have a shorter windows of time between when they present as risky, premalignant lesions (evolving slowly, removable, and treatable), when they are early, localized cancers (more rapidly evolving, but still removable and treatable) and when they evolve to metastatic disease (rapidly evolving, difficult to treat, low survival).

The evolution of a neoplasm is a stochastic process and one that is closely related to the particular environmental pressures that exist at the cellular and tissue levels. Nearly all patients with BE are treated medically or surgically to reduce the effects of reflux, and those that are considered to be at high risk for the development of EA undergo endoscopic therapies such as radiofrequency ablation or endoscopic mucosal resection to destroy or remove the risky Barrett's epithelia [92]. However, ~90 % of EAs are diagnosed in patients who are not in surveillance and were never suspected to have BE [38, 41], 40 % of whom don't even report symptoms of reflux [54, 93], and would never have a reason to be treated for GERD. Thus, compared to diagnosed BE patients who are in surveillance and being treated for reflux, the environment of the esophagus in the majority of the patients in which EAs develop may be very different. Is the neoplastic evolution we are characterizing in BE patients in surveillance different from that occurring in a patient who develops EA silently? A detailed comparison of the EAs arising in these two populations would be a reasonable place to start addressing this question.

Back in 1976, Nowell suggested that perhaps the best way to deal with adaptability of the evolving neoplasm would be to use adaptive system against it—the immune system. "One may ultimately have to consider each advanced malignancy

as an individual therapeutic problem after as many cells as possible have been eliminated through the nonspecific modalities of surgery, radiation, and chemotherapy. Then, perhaps, immunotherapy becomes a leading candidate for the easiest means of destroying the remainder of the neoplastic clone [1]." Recent trials of compounds to activate the body's immune response have yielded some remarkable successes (see [94] for review). The adaptive immune system has been evolving for the past 500 million years [95]; perhaps it is now time to utilize it as an improved treatment modality.

The standard of care that allows sampling of a developing neoplasm over time and space has made BE an excellent model system for studying the process of how cancer develops and evolves in vivo. Many very basic questions concerning BE and progression to EA remain: Why do nearly all cell populations in the Barrett's epithelium have genomic alterations? How and why do the genomes of nonprogressors remain stable? In patients who do progress to EA, how is the rampant instability that generates variant clones initiated? It is hoped that the answers to these and related questions in other systems will promote the development of more effective early detection and risk assessment strategies to augment the Sisyphean task of reducing the morbidity and mortality of treating advanced cancers.

References

1. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194(4260):23–8.
2. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977;74(12):5463–7.
3. Aktipis CA, Kwan VS, Johnson KA, et al. Overlooking evolution: a systematic analysis of cancer relapse and therapeutic resistance research. PLoS One. 2011;6(11):e26100. doi:10.1371/journal.pone.0026100.
4. Croswell JM, Ransohoff DF, Kramer BS. Principles of cancer screening: lessons from history and study design issues. Semin Oncol. 2010;37(3):202–15. doi:10.1053/j.seminoncol.2010.05.006.
5. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988;319(9):525–32.
6. Vogelstein B, Papadopoulos N, Velculescu VE, et al. Cancer genome landscapes. Science. 2013;339(6127):1546–58. doi:10.1126/science.1235122.
7. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860–921. doi:10.1038/35057062.
8. Dulbecco R. A turning point in cancer research: sequencing the human genome. Science. 1986;231(4742):1055–6.
9. Leiserson MD, Vandin F, Wu HT, et al. Pan-cancer network analysis identifies combinations of rare somatic mutations across pathways and protein complexes. Nat Genet. 2015;47(2):106–14. doi:10.1038/ng.3168.
10. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012;366(10):883–92. doi:10.1056/NEJMoa1113205.
11. de Bruin EC, McGranahan N, Mitter R, et al. Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. Science. 2014;346(6206):251–6. doi:10.1126/science.1253462.

12. Zhang J, Fujimoto J, Zhang J, et al. Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. Science. 2014;346(6206):256–9. doi:10.1126/science.1256930.
13. Sottoriva A, Kang H, Ma Z, et al. A Big Bang model of human colorectal tumor growth. Nat Genet. 2015;47(3):209–16. doi:10.1038/ng.3214.
14. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. Nature. 2011;472(7341):90–4. doi:10.1038/nature09807.
15. Murugaesu N, Wilson GA, Birkbak NJ, et al. Tracking the genomic evolution of esophageal adenocarcinoma through neoadjuvant chemotherapy. Cancer Discov. 2015. doi:10.1158/2159-8290.CD-15-0412.
16. Li X, Galipeau PC, Paulson TG, et al. Temporal and spatial evolution of somatic chromosomal alterations: a case-cohort study of Barrett's esophagus. Cancer Prev Res (Phila). 2014;7(1):114–27. doi:10.1158/1940-6207.CAPR-13-0289.
17. Agrawal N, Jiao Y, Bettegowda C, et al. Comparative genomic analysis of esophageal adenocarcinoma and squamous cell carcinoma. Cancer Discov. 2012;2(10):899–905. doi:10.1158/2159-8290.CD-12-0189.
18. Dulak AM, Stojanov P, Peng S, et al. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. Nat Genet. 2013;45(5):478–86. doi:10.1038/ng.2591.
19. Nones K, Waddell N, Wayte N, et al. Genomic catastrophes frequently arise in esophageal adenocarcinoma and drive tumorigenesis. Nat Commun. 2014;5:5224. doi:10.1038/ncomms6224.
20. Streppel MM, Lata S, DelaBastide M, et al. Next-generation sequencing of endoscopic biopsies identifies ARID1A as a tumor-suppressor gene in Barrett's esophagus. Oncogene. 2014;33(3):347–57. doi:10.1038/onc.2012.586.
21. Weaver JM, Ross-Innes CS, Shannon N, et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet. 2014;46(8):837–43. doi:10.1038/ng.3013.
22. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. Nature. 2013;500(7463):415–21. doi:10.1038/nature12477.
23. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202–9. doi:10.1038/nature13480.
24. Wang K, Yuen ST, Xu J, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014;46(6):573–82. doi:10.1038/ng.2983.
25. Wang Y, Waters J, Leung ML, et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature. 2014;512(7513):155–60. doi:10.1038/nature13600.
26. Paulson TG, Reid BJ. Focus on Barrett's esophagus and esophageal adenocarcinoma. Cancer Cell. 2004;6(1):11–6.
27. Reid BJ, Li X, Galipeau PC, et al. Barrett's oesophagus and oesophageal adenocarcinoma: time for a new synthesis. Nat Rev Cancer. 2010;10(2):87–101. doi:10.1038/nrc2773.
28. Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. Am J Gastroenterol. 2008;103(3):788–97.
29. Howlader N, Noone AM, Krapcho M, et al. (2014). SEER cancer statistics review, 1975-2011. from National Cancer Institute http://seer.cancer.gov/csr/1975_2011/.
30. Ostrowski J, Mikula M, Karczmarski J, et al. Molecular defense mechanisms of Barrett's metaplasia estimated by an integrative genomics. J Mol Med. 2007;85(7):733–43.
31. Dixon J, Strugala V, Griffin SM, et al. Esophageal mucin: an adherent mucus gel barrier is absent in the normal esophagus but present in columnar-lined Barrett's esophagus. Am J Gastroenterol. 2001;96(9):2575–83.
32. Tobey NA, Argote CM, Kav T, et al. Anion transport in human squamous and Barrett's esophageal epithelium. Gastroenterology. 2005;128:A234.
33. Jovov B, Van Itallie CM, Shaheen NJ, et al. Claudin-18: a dominant tight junction protein in Barrett's esophagus and likely contributor to its acid resistance. Am J Physiol Gastrointest Liver Physiol. 2007;293(6):G1106–13.

34. Leedham SJ, Preston SL, McDonald SA, et al. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. Gut. 2008;57(8):1041–8.
35. McDonald SA, Lavery D, Wright NA, et al. Barrett oesophagus: lessons on its origins from the lesion itself. Nat Rev Gastroenterol Hepatol. 2015;12(1):50–60. doi:10.1038/nrgastro.2014.181.
36. Wang X, Ouyang H, Yamamoto Y, et al. Residual embryonic cells as precursors of a Barrett's-like metaplasia. Cell. 2011;145(7):1023–35. doi:10.1016/j.cell.2011.05.026.
37. Xian W, Ho KY, Crum CP, et al. Cellular origin of Barrett's esophagus: controversy and therapeutic implications. Gastroenterology. 2012;142(7):1424–30. doi:10.1053/j.gastro.2012.04.028.
38. Vaughan TL, Fitzgerald RC. Precision prevention of oesophageal adenocarcinoma. Nat Rev Gastroenterol Hepatol. 2015;12(4):243–8. doi:10.1038/nrgastro.2015.24.
39. Bhat S, Coleman HG, Yousef F, et al. Risk of malignant progression in Barrett's esophagus patients: results from a large population-based study. J Natl Cancer Inst. 2011;103(13):1049–57. doi:10.1093/jnci/djr203.
40. de Jonge PJ, van Blankenstein M, Looman CW, et al. Risk of malignant progression in patients with Barrett's oesophagus: a Dutch nationwide cohort study. Gut. 2010;59(8):1030–6. doi:10.1136/gut.2009.176701.
41. Hvid-Jensen F, Pedersen L, Drewes AM, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011;365(15):1375–83. doi:10.1056/NEJMoa1103042.
42. Welch HG, Black WC. Overdiagnosis in cancer. J Natl Cancer Inst. 2010;102(9):605–13. doi:10.1093/jnci/djq099.
43. Levine DS, Blount PL, Rudolph RE, et al. Safety of a systematic endoscopic biopsy protocol in patients with Barrett's esophagus. Am J Gastroenterol. 2000;95(5):1152–7.
44. Varghese S, Lao-Sirieix P, Fitzgerald RC. Identification and clinical implementation of biomarkers for Barrett's esophagus. Gastroenterology. 2012;142(3):435–41. doi:10.1053/j.gastro.2012.01.013. e432.
45. Carter SL, Cibulskis K, Helman E, et al. Absolute quantification of somatic DNA alterations in human cancer. Nat Biotechnol. 2012;30(5):413–21. doi:10.1038/nbt.2203.
46. Rabinovitch PS, Reid BJ, Haggitt RC, et al. Progression to cancer in Barrett's esophagus is associated with genomic instability. Lab Invest. 1988;60(1):65–71.
47. Barrett MT, Sanchez CA, Prevo LJ, et al. Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Gen. 1999;22(1):106–9.
48. Blount PL, Meltzer SJ, Yin J, et al. Clonal ordering of 17p and 5q allelic losses in Barrett dysplasia and adenocarcinoma. Proc Natl Acad Sci U S A. 1993;90(8):3221–5.
49. Neshat K, Sanchez CA, Galipeau PC, et al. Barrett's esophagus: a model of human neoplastic progression. Cold Spring Harb Symp Quant Biol. 1994;59:577–83.
50. Maley CC, Galipeau PC, Finley JC, et al. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. Nat Genet. 2006;38(4):468–73.
51. Galipeau PC, Li X, Blount PL, et al. NSAIDs modulate CDKN2A, TP53, and DNA content risk for future esophageal adenocarcinoma. PLoS Med. 2007;4:e67.
52. Kostadinov RL, Kuhner MK, Li X, et al. NSAIDs modulate clonal evolution in Barrett's esophagus. PLoS Genet. 2013;9(6):e1003553. doi:10.1371/journal.pgen.1003553.
53. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012;481(7381):306–13. doi:10.1038/nature10762.
54. Lagergren J, Bergstrom R, Lindgren A, et al. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. N Engl J Med. 1999;340(11):825–31.
55. Whiteman DC, Sadeghi S, Pandeya N, et al. Combined effects of obesity, acid reflux and smoking on the risk of adenocarcinomas of the oesophagus. Gut. 2008;57(2):173–80.
56. Wu AH, Tseng CC, Bernstein L. Hiatal hernia, reflux symptoms, body size, and risk of esophageal and gastric adenocarcinoma. Cancer. 2003;98(5):940–8.
57. Anderson AR, Weaver AM, Cummings PT, et al. Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment. Cell. 2006;127(5):905–15. doi:10.1016/j.cell.2006.09.042.

58. Jenkins GJ, Cronin J, Alhamdani A, et al. The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS. Mutagenesis. 2008;23(5):399–405.
59. Grisham MB, Jourd'heuil D, Wink DA. Review article: chronic inflammation and reactive oxygen and nitrogen metabolism--implications in DNA damage and mutagenesis. Aliment Pharmacol Ther. 2000;14 Suppl 1:3–9.
60. Sihvo EI, Ruohtula T, Auvinen MI, et al. Simultaneous progression of oxidative stress and angiogenesis in malignant transformation of Barrett esophagus. J Thorac Cardiovasc Surg. 2003;126(6):1952–7.
61. Foster JM, Oumie A, Togneri FS, et al. Cross-laboratory validation of the OncoScan(R) FFPE assay, a multiplex tool for whole genome tumour profiling. BMC Med Genomics. 2015;8(1):5. doi:10.1186/s12920-015-0079-z.
62. Prentice RL. A case-cohort design for epidemiologic cohort studies and disease prevention trials. Biometrika. 1986;73:1–11.
63. Cheng H, Bjerknes M, Amar J. Methods for the determination of epithelial cell kinetic parameters of human colonic epithelium isolated from surgical and biopsy specimens. Gastroenterology. 1984;86(1):78–85.
64. Yatabe Y, Tavare S, Shibata D. Investigating stem cells in human colon by using methylation patterns. Proc Natl Acad Sci U S A. 2001;98(19):10839–44.
65. Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. Nature. 2010;463(7283):899–905. doi:10.1038/nature08822.
66. Li X, Galipeau PC, Sanchez CA, et al. Single nucleotide polymorphism-based genome-wide chromosome copy change, loss of heterozygosity, and aneuploidy in Barrett's esophagus neoplastic progression. Cancer Prev Res (Phila). 2008;1(6):413–23. doi:10.1158/1940-6207.CAPR-08-0121.
67. Fleming TR, Powers JH. Biomarkers and surrogate endpoints in clinical trials. Stat Med. 2012;31(25):2973–84. doi:10.1002/sim.5403.
68. Prentice RL. Surrogate endpoints in clinical trials: definition and operational criteria. Stat Med. 1989;8(4):431–40.
69. Montgomery E, Bronner MP, Goldblum JR, et al. Reproducibility of the diagnosis of dysplasia in Barrett esophagus: a reaffirmation. Hum Pathol. 2001;32(4):368–78.
70. Odze RD. What the gastroenterologist needs to know about the histology of Barrett's esophagus. Curr Opin Gastroenterol. 2011;27(4):389–96. doi:10.1097/MOG.0b013e328346f551.
71. Reid BJ, Haggitt RC, Rubin CE, et al. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. Hum Pathol. 1988;19(2):166–78.
72. Reid BJ, Levine DS, Longton G, et al. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol. 2000;95(7):1669–76.
73. Schnell TG, Sontag SJ, Chejfec G, et al. Long-term nonsurgical management of Barrett's esophagus with high-grade dysplasia. Gastroenterology. 2001;120(7):1607–19.
74. Sharma P, Falk GW, Weston AP, et al. Dysplasia and cancer in a large multicenter cohort of patients with Barrett's esophagus. Clin Gastroenterol Hepatol. 2006;4(5):566–72.
75. Weston AP, Sharma P, Topalovski M, et al. Long-term follow-up of Barrett's high-grade dysplasia. Am J Gastroenterol. 2000;95(8):1888–93.
76. Orlando RC. Mucosal defense in Barrett's esophagus. In S R, Sharma P, editors. Barrett's esophagus and esophageal adenocarcinoma. 2nd ed. Oxford, UK: Blackwell Publishing, Ltd.; 2006. p. 60–72.
77. Bandla S, Pennathur A, Luketich JD, et al. Comparative genomics of esophageal adenocarcinoma and squamous cell carcinoma. Ann Thorac Surg. 2012;93(4):1101–6. doi:10.1016/j.athoracsur.2012.01.064.
78. Gu J, Ajani JA, Hawk ET, et al. Genome-wide catalogue of chromosomal aberrations in barrett's esophagus and esophageal adenocarcinoma: a high-density single nucleotide polymorphism array analysis. Cancer Prev Res (Phila). 2010;3(9):1176–86. doi:10.1158/1940-6207.CAPR-09-0265.

79. Nancarrow DJ, Handoko HY, Smithers BM, et al. Genome-wide copy number analysis in esophageal adenocarcinoma using high-density single-nucleotide polymorphism arrays. Cancer Res. 2008;68(11):4163–72.
80. Maley CC, Galipeau PC, Li X, et al. Selectively advantageous mutations and hitchhikers in neoplasms: p16 lesions are selected in Barrett's esophagus. Cancer Res. 2004;64(10):3414–27.
81. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell. 2011;144(1):27–40. doi:10.1016/j.cell.2010.11.055.
82. Li X, Paulson TG, Galipeau PC, et al. Assessment of esophageal adenocarcinoma risk using somatic chromosome alterations in longitudinal samples in Barrett's esophagus. Cancer Prev Res (Phila). 2015. doi:10.1158/1940-6207.capr-15-0130.
83. Kandoth C, McLellan MD, Vandin F, et al. Mutational landscape and significance across 12 major cancer types. Nature. 2013;502(7471):333–9. doi:10.1038/nature12634.
84. Levine DS, Haggitt RC, Blount PL, et al. An endoscopic biopsy protocol can differentiate high-grade dysplasia from early adenocarcinoma in Barrett's esophagus. Gastroenterology. 1993;105(1):40–50.
85. Kadri SR, Lao-Sirieix P, O'Donovan M, et al. Acceptability and accuracy of a non-endoscopic screening test for Barrett's oesophagus in primary care: cohort study. BMJ. 2010;341:c4372. doi:10.1136/bmj.c4372.
86. Ross-Innes CS, Debiram-Beecham I, O'Donovan M, et al. Evaluation of a minimally invasive cell sampling device coupled with assessment of trefoil factor 3 expression for diagnosing Barrett's esophagus: a multi-center case-control study. PLoS Med. 2015;12(1):e1001780. doi:10.1371/journal.pmed.1001780.
87. Bridson EY, Gould GW. Quantal microbiology. Lett Appl Microbiol. 2000;30(2):95–8.
88. Buks E, Schuster R, Heiblum M, et al. Dephasing in electron interference by a "which-path" detector. Nature. 1998;391(6670):871–4.
89. Rothwell PM, Fowkes FG, Belch JF, et al. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. Lancet. 2011;377(9759):31–41. doi:10.1016/S0140-6736(10)62110-1.
90. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345(6274):458–60.
91. Baca SC, Prandi D, Lawrence MS, et al. Punctuated evolution of prostate cancer genomes. Cell. 2013;153(3):666–77. doi:10.1016/j.cell.2013.03.021.
92. Wang KK. Current Strategies in the management of Barrett's esophagus. Curr Gastroenterol Rep. 2005;7(3):196–201.
93. Farrow DC, Vaughan TL, Sweeney C, et al. Gastroesophageal reflux disease, use of H2 receptor antagonists, and risk of esophageal and gastric cancer. Cancer Causes Control. 2000;11(3):231–8.
94. Mueller KL. Cancer immunology and immunotherapy. Realizing the promise. Introduction. Science. 2015;348(6230):54–5. doi:10.1126/science.348.6230.54.
95. Flajnik MF, Kasahara M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet. 2010;11(1):47–59. doi:10.1038/nrg2703.

Chapter 12
Genomics of Esophageal Cancer and Biomarkers for Early Detection

Mark Pusung, Sebastian Zeki, and Rebecca Fitzgerald

Introduction

Carcinoma of the esophagus is the eighth leading cause of cancer-related deaths worldwide [1]. With nearly 456,000 new cases diagnosed in 2012, its incidence has increased rapidly since the 1970s [2, 3]. Most patients are diagnosed between the ages 50 and 60 years old with men almost four times more likely to be affected. Esophageal cancer is generally classified into two types: Esophageal Squamous Cell Carcinoma (OSCC) and Esophageal Adenocarcinoma (OAC). Smoking and alcohol abuse are commonly associated with OSCC, while reflux of acid and bile is the main risk factor for OAC via a precursor lesion Barrett's esophagus (BO) [4]. In the western world, over half of all esophageal cancers are of the adenocarcinoma type [1], which has a 5-year survival rate of less than 20 %. Its high morbidity and mortality rates are attributed to the late presentation of symptoms and the current lack of effective risk stratification clinical practices [5]. The continuous increase in frequency combined with poor prognosis makes OAC a global health concern.

Uncovering the cellular processes and structure of many human cancers has been a core focus of modern methods capable of genome-wide analysis. With the ultimate goal of enhancing clinical practice, different platforms have been used to achieve a more comprehensive investigation of the diverse genetic alterations driving oncogenesis. Elucidation of aberrations such as copy number variations, genetic mutations, and epigenetic changes has remained critical in understanding human malignancies. In recent years, detailed characterization of tumors has complemented histological classification of malignancies in different organs with greater detail. In some cases, results from genome-wide studies have been the basis for reclassification and organization of malignancies into subtypes [6]. Further elucidation of the

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ordering of key mutations in the disease progression will have significant impact on risk stratification and patient surveillance. In order to ascertain biomarkers to diagnose and/or predict the progression of BO to adenocarcinoma, a better, detailed understanding of molecular pathogenesis is required.

Landscape of Genomic Alterations in Esophageal Adenocarcinoma

Accumulation of aberrant global and local chromosomal events resulting in aneuploidy, chromosomal rearrangements, tumor suppressor inactivation, and activation of oncogenes are frequently associated with progression to esophageal adenocarcinoma. In recent years, high-throughput sequencing techniques have resulted in better characterization of these events as well as the discovery of new targets.

Genetic Aberrations in Cell Signaling Pathways

EGFR and HER2

Dysregulation at the receptor level reflects phenotypic abnormalities on cell growth and proliferation. Amplifications and activating mutations of several membrane-bound receptor tyrosine kinases (RTKs) have been reported in several cancers [7]. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) are examples of two well-studied RTKs. EGFR and its corresponding ligands EGF and transforming growth factor-alpha (TGF-α) are overexpressed in OAC [8]. Activating mutations in the EGFR kinase domain have also been characterized. Specifically, sequencing of the *EGFR* gene has revealed that recurrent missense mutations and in-frame deletions occur early in the transformation of the esophageal epithelium [9]. A meta-analysis of 33 studies found that prevalence of *HER2* amplification and/or overexpression was 24 % (95 % confidence interval [CI]: 15–36 %) in BO and 26 % (95 % CI: 19–34 %) in OAC [10]. More importantly, the abundant expression of *HER2* in esophageal carcinogenesis seems to occur in later stages of progression to malignancy [11] and is correlated with poor prognosis [12] (see Table 12.1 for a list of commonly amplified genes in OAC). A recent study on dimerization patterns of these receptors in OAC revealed that *HER2* and *EGFR* are frequently coamplified and preferentially dimerize with one another [13]. Elucidation of such biochemical patterns has great implications on therapeutic regimens, as drugs specifically designed to target only one receptor are less likely to have dramatic effect on cancers driven by heterodimers.

Table 12.1 Regions with frequent copy number gains and commonly amplified genes in esophageal adenocarcinoma

Cytoband	Gene	Role	Reference
17q12	*HER2*	Receptor tyrosine kinase	[13, 21, 35, 68]
7p11.2	*EGFR*	Receptor tyrosine kinase	[13]
19q12	*CCNE1*	G1/S cyclin	[13]
11913.3	*CCND1*	G1/S cyclin	
8p23.1	*GATA4*	Oncogene/transcription factor	[21]
12p12	*KRAS*	GTPase/signal transduction	[21]
8q24.21	*C-MYC*	Oncogene/transcription factor	[21, 75]
3q26.32	*PIK3CA*	Signal transduction	[21]
14q21.1	*HNF3-alpha*	Transcription factor	[21]
12q15	*DYRK2*	Cytoplasmic tyrosine kinase	[21]
20q.13.12	*AIB1*	Transcriptional coactivator	[21]
7q31.2	*MET*	Kinase receptor	[13, 35]
13q22.1	*KLF12*	Transcription factor	[75]
8p11.22/10q26.13	*FGFR1/2*	Receptor tyrosine kinase	[35]

Transforming Growth Factor-β

The transforming growth factor-β pathway regulating growth inhibition and suppression of genomic instability requires the transcription factors SMAD proteins and Runt domain transcription factor 3 (RUNX3) [14]. Dysregulation of this pathway promotes proliferation and invasion in OAC. Inactivating mutations and diminished expression of TGF-β1 receptor type II has been reported in OAC where the loss of TGF-β signaling prevents cell cycle arrest and promotes invasion [15]. In addition, homozygous deletions of the downstream effector *SMAD4* has been shown in OAC cell lines and xenograft models [16]. Recently, comprehensive analysis of the TGF-B-SMAD signaling pathway in 149 chemo naive OAC tumors with matched normal samples and available whole-exome sequencing data revealed that *SMAD4* is the most recurrently altered gene. Specifically, the authors reported that *SMAD4* had a mutation frequency of 8 %, while copy number loss was observed in 34 % of the cases [17]. In an in-depth assessment of the ordering of somatic mutations in OAC, Weaver and colleagues provided evidence for the stage-specific occurrence of *SMAD4* alterations. Whole-genome and amplicon sequencing of 112 OACs and subsequent comparative analysis of 109 nonmalignant biopsy samples showed that although nondysplastic BO and OAC generally share a common mutational landscape, *SMAD4* mutations are confined in the malignant stage of the disease [18]. In a cohort of 205 OACs, evaluation by immunohistochemistry (IHC) revealed that Smad4 loss is correlated with increased postoperative recurrence. In this study, patients harboring Smad4 expression had longer time to recurrence (23 months) and overall survival (OS: 22 months) than patients with *Smad*-deficient tumors (13 months and OS: 16 months, respectively) [19]. In vitro, exogenous *RUNX3* reactivates the TGF-β-induced apoptosis in OAC cells lacking *RUNX3* [14].

A recent meta-analysis of nine studies consisting of 558 patients in total showed that downregulation of *RUNX3* by promoter methylation was significantly higher in OAC than BO. Like *SMAD4*, decreased *RUNX3* expression was correlated with poor overall survival (Hazard Ratio [HR] = 4.31, 95 % CI = 2.57–7.37) [20].

c-myc

The oncogenic driver *c-myc* is a transcription factor that is essential for the expression of genes required for cell proliferation. *c-myc* amplification is observed to be an oncogenic event co-occurring with other cancer drivers such as *HER2* amplification [13, 21]. Alteration in the *c-myc* gene seems to be stage specific as well. In an assessment of 43 esophagectomy specimens from patients with low-grade dysplasia ($N=23$), high-grade dysplasia ($N=24$), and invasive adenocarcinoma ($N=39$), the incidence of *c-myc* amplification increased with worsening histopathology [22]. Two separate studies also investigated secondary genomic alteration co-occurring with *c-myc* amplification. In one study, the frequency of 7p12 (EGFR), 8q24 (*c-myc*), and 20q13 copy gains increased with advanced stages of the disease. Another study consisting of 84 primary resected OACs reported increased VEGF and COX-2 expression in *c-myc* amplified samples [23]. Activation of VEGF leads to vascularization of adenocarcinomas while COX-2 permits the proliferative potential in OAC. Lagorce et al. assessed COX2 expression BO ($n=15$), BO plus dysplasia ($n=17$), and OAC ($n=66$) by immunohistochemistry and found that more than 90 % of samples had COX2 upregulation suggesting its alteration in premalignant condition [24]. Immunnohistochemical analysis of tissue microarrays from 154 OAC patients showed overexpression of COX-2 and VEGF in 27 and 54 % of cases, respectively [25].

Abnormalities in DNA Repair and Cell Cycle Genes

TP53

The tumor suppressor *TP53* is one of the most frequently mutated gene in all cancer types [26]. Inactivation of *TP53* and/or its gene product p53 lead to dysregulated cell cycle checkpoints and subsequent accumulation of unrepaired genetic damages. Deletion of one allele followed by an inactivating mutation in the other allele, or vice versa, can lead to complete loss of p53 function. In an effort to establish the importance of p53 aberration in esophageal neoplastic progression, Dolan et al. conducted mutational analysis on normal and malignant tissues from patients undergoing esophagectomy ($N=30$) and premalignant samples from those undergoing routine surveillance for BO ($N=48$). Thirty-three percent (10 out of the 30) patients had *TP53* mutations, which were more common in carcinomas that are well differentiated. From the Barrett's group, two patients had *TP53* mutations with one progressing to malignancy during the study. Overall, patients without *TP53* mutations remained stable and did not develop high-grade dysplasia (HGD) or OAC [27].

Indeed, p53 loss of heterozygosity (LOH) and mutations occur even in the premalignant stages of the disease [28]. In one study, p53 overexpresssion was reported to be present in 60 % of patients with HGD but only 12 % in low-grade dysplasia (LGD) cases [29]. However, in large cohort ($N=112$ OACs; $N=66$ nondysplastic BOs; $N=43$ high-grade dysplasia), whole-genome and amplicon sequencing revealed *TP53* mutations occur only in cases that are defined histopathologically as being high grade [18]. Analysis of 145 OACs by exome and whole-genome sequencing revealed that 83 % of the cases had either *TP53* mutation or copy number loss [17]. Findings from these studies show that p53 aberrations can be used as a marker for neoplastic transformation. The use of p53 as a biomarker in prospective studies will be discussed in the latter part of this chapter.

CDKN2A

The *CDKN2A* gene encodes the cell cycle regulator p16, which inhibits the cyclin-dependent kinases, such as CDK4 and CDK6, integral to G to S phase progression. *CDKN2A* LOH and mutation frequently precede p53 abnormalities [30]. It has been shown that *CDKN2A* and alternate reading frame $p14^{ARF}$ lesions (LOH at microsatellite D9S925, sequence mutations, and promoter methylation) are the most common epigenetic and genetic aberrations in early stages of BO and are carried on to advanced stages of the disease [31]. Epigenetic modification, however, seems to be the primary mechanism leading to the loss of *CDKN2A* expression, with inactivating mutation occurring less frequently. A study of surgically resected tissues ($N=54$ primary tumor, $N=24$ associated Barrett's epithelia, $N=43$ matched histologically normal, or $N=22$ gastric epithelia from distant resection margins) from 54 patients showed that *CDKN2A* mutations were only present in 2 % of the cases while promoter methylation occurred in 77 % of associated Barrett's epithelia and in 85 % (18/21) OAC cases [32]. This is consistent with a study by Bian et al. in which *CDKN2A* methylation was the predominant mechanism of inactivation in 82 % of adenocarcinomas and 30 % of premalignant tissue samples analyzed [33]. Examination of $p14^{ARF}$ events in BO and OAC also revealed reduction of tumor suppressor $p14^{ARF}$ due to promoter methylation in 57 out 76 patients with OAC [34]. In a more recent study, in-depth analysis of somatic copy-number alterations in 296 OAC samples showed that focal deletions are recurrent in the chromosomal region 9p containing *CDKN2A* [35] (see Table 12.2 for a list of commonly deleted genes in OAC).

Cyclins and Cyclin-Dependent Kinases

Tight regulation of cell cycle progression is a key to homeostatic cell growth and proliferation. Dysregulation of the key players in this mechanism can lead to tumor growth [36]. Cyclins are particularly unique in that their expression levels vary during cell cycle. In normal cells, cyclin B increases as cells approach mitosis.

Table 12.2 Regions with frequent copy number loss and commonly deleted genes in esophageal adenocarcinoma

Cytoband	Gene	Role	Reference
17p13.1	*TP53*	Tumor suppressor	
9p21.3	*CDKN2A (p16)*	Tumor suppressor	[16, 75]
18q21.1	*SMAD4*	Transcription factor	[16, 75]
16p22.1	*CDH3*	Cell–cell adhesion	[16]
16q22.1	*CDH1*	Cell–cell adhesion	[16]
2q32.1	*ITGAV*	Adhesion/signal transduction	[16]
21q22.12	*RUNX1*	Transcription factor	[16, 35]
3p14.2	*FHIT*	Tumor suppressor	[75]
16q23.1-2	*WWOX*	Transcription factor	[81]
5q22.2	*APC*	Tumor suppressor	[27]
18q21.2	*DCC*	Tumor suppressor	[27]
10q23.31	*PTEN*	Tumor suppressor	

Semiquantitative determination of cyclin B expression cells also showed a sustained overexpression from dysplasia to carcinoma [37]. Cyclin D1 amplification was reported to be as high as 64 % in OAC cases [38]. In a study by Morgan et al. *CCND1* gene amplification was correlated with overexpression of Cyclin D1 and amplification of p53 modulator *MDM2* [39]. The S phase-specific Cyclin E encoded by the *CCNE1* gene was observed to be amplified in 22 out of 116 OAC samples. In-depth analysis of patients who progressed to malignancy showed that its expression increases from nondysplastic stage to high-grade dysplasia suggesting its role in the early phase of malignant transformation [40]. *CCNE1* amplification is also a frequent secondary alteration in HER2-amplified gastroesophageal adenocarcinoma potentially conferring drug resistance [13]. Complex formation of cyclins with their respective kinases is an integral component of cell cycle. 7q21 and 12q14 contain the cyclin-dependent kinases *CDK4* and *CDK6,* respectively, and these regions have been reported to be amplified in BO and OAC [35, 41]. *CDK4* and *CDK6* loci amplification frequency revealed by whole-exome sequencing of OAC tumors ($N = 149$) was 3 and 17 %, respectively [17].

Dysregulation of Wnt Signaling and Cell Adhesion Genes

Catenins and the transmembrane protein E-cadherin are required for the maintenance of adherens junctions binding epithelial cells together. Loss of E-cadherin leads to the disruption of the adhesion complex, loss of contact inhibition and, consequently, uncontrolled cell growth [42]. For this reason, interference of cell adhesion is considered an early event in oncogenesis [43]. Reduced expression of E-cadherin is observed in BO and in OAC where significant absence of E-cadherin is correlated with poor cell differentiation and survival [44]. Although LOH and epigenetic silencing of the E-cadherin locus is frequent, mutations in E-cadherin are

not common. Dulak and colleagues reported frequency of mutation and loss of only 3 and 4 %, respectively, in 149 OAC tumors [17]. In one study involving cadherin–catenin complex, more than 75 % (60/80) of OAC and lymph node samples showed reduction of E-cadherin, alpha-catenin, and beta-catenin expression. In this study, low expression levels of all three proteins correlated with high tumor grade and shorter patient survival [45]. This correlation is consistent with a separate study where 42 out 59 OAC displayed reduced or absent membranous E-cadherin [43].

Overexpression of the receptor tyrosine kinase Ephrin B3 (Eph B3) inhibits cell growth and its activity is reduced in OAC. Simultaneous expression of Eph B3 and translocation of E-cadherin to the membrane correlated inversely to tumor stage [46].

The Wnt signaling pathway is responsible for promoting the intestinal architecture [47]. It downregulates the expression of cadherins [48] and upregulates the expression of the proto-oncogene beta-catenin [49]. Since OAC develops from an intestinal metaplasia background, Moyes et al. have explored whether aberrant Wnt signaling is activated in the development of BO and/or dysplasia. The authors reported that high-grade dysplasia was correlated with nuclear localization of beta-catenin and upregulated expression of Wnt target genes *cyclin D1, Sox-9,* and *c-myc*. This activation of the Wnt signaling pathway was not observed in nondysplastic tissue samples [50]. Increased activity of the Wnt pathway can also be due to the increased expression of the Wnt ligand WNT2. Clement et al. reported that OAC samples had significantly higher levels of WNT2 mRNA when compared with normal and nonmalignant tissue samples. In addition, cancer and dysplastic samples displayed reduction in levels of Wnt inhibitors SFRP1 and SFRP2 [51].

Gross Chromosomal Anomalies

As well as point mutations and focal events there are also large-scale chromosomal alterations. For example, alterations in ploidy status (aneuploidy/tetraploidy) and regional DNA fragility constitute gross chromosomal abnormalities in malignant transformation [52].

Aneuploidy and Microsatellite Instability

In an early study by Reid and colleagues exploring the relationship between DNA content and histological classification, all 64 patients under cancer surveillance had evidence of aneuploidy regardless of the extent of their dysplasia [53]. In a retrospective study of 23 patients who developed adenocarcinoma, analysis of ploidy status by flow cytometry showed strong association between aneuploidy/tetraploidy and poor prognosis with 11 out of the 16 patients harboring aneuploid tumors not surviving [54, 55]. Detection of DNA content using the more sensitive image cytometry on sections also revealed increased frequency and severity of aneuploidy

in advanced grades of dysplasia. In this study, a total of 187 cases ($N=34$ controls [normal gastrointestinal mucosa]; $N=66$ BO with intestinal metaplasia [IM]; $N=22$ LGD; $N=22$ HGD; $N=43$ OAC) were examined. All OAC samples had aneuploid cell populations and significant increase in DNA content was observed in 5 % of IM, 32 % of LGD, and 80 % of HGD cases [55]. Comprehensive spatial analysis of DNA ploidy abnormalities in BO crypts revealed that basal crypt cells were aneuploid in 37 % of nondysplastic BO (NDBO) and 73 % of LGD samples while superficial crypt cells were diploid in NDBO and only aneuploid in 50 % of LGD cases suggesting an upward cellular DNA content aberration and dysplasia in the basal crypt representing the earliest indication of dysplastic change [56]. Chromosomal rearrangements and shattering usually clustered around certain areas of the genome, known as *chromothripsis,* as evidence of extreme genomic instability has also been reported in OAC. Using a combination of whole-genome sequencing (WGS) and single-nucleotide polymorphism array, Nones et al., observed chromothriptic events in 32 % ($N=123$) of cases analyzed [57].

Microsatellites are repeated oligonucleotides of usually 2–5 bp that are vulnerable to DNA replication errors [58]. In nondysplastic cells, mismatch repair (MMR) genes, which include *MLH1, MSH2, MSH6, PMS1,* and *PMS2*, are responsible for correcting mistakes in DNA base pairing during replication. Defects in MMR and microsatellite instability (MSI) have been reported as crucial to neoplastic progression in many cancers [59]. Reports of MSI in BO and OAC have been variable, making the roles of, and the relationship between, MMR anomalies and MSI still left undefined [60]. OAC tumors are classified as microsatellite-stable (MSS), low-level MSI (MSI-L), and high-level MSI (MSI-H) depending on the number of MSI markers used. In one study, 27 primary OAC cases using a 15-panel MSI marker showed that two-thirds of the tumors harbored stable MSI while the remainder were only MSI-L. Moreover, it is important to note that only 6 out of the 27 tumor samples showed altered expression of MLH1 and MSH2, further establishing the lack of association between presence of MSI and reduced activity of the MMR machinery [61]. In a flow cytometry-based study focusing specifically on five microsatellite loci, 22 % (8/36) of the OAC patients had MSI-H at one or more of the evaluated loci occurring in both diploid and aneuploid cell populations [62]. A separate group, however, reported a lower frequency of MSI (<8 %) in 139 loci in 17 cases of OAC [63]. This is consistent with two recent studies reporting a low prevalence rate of MSI-H. Using a five-panel marker (BAT-25, BAT-26, D5S346, D17S250, and D2S123), Farris et al. observed high MSI in only ~7 % of OAC cases ($N=76$). A much lower frequency, however, was reported by Dulak and colleagues when ten biomarkers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT4, D18S55, D18S56, D18S67, and D18S487) were utilized. Only 4 out 149 samples analyzed by whole-exome sequencing were classified as MSI-H tumors [17].

Since MSI is an indication of genomic instability, Khara et al. examined the association between extent of mutational load (ML), defined as the presence of LOH and the number of MSI mutations, and histological classification [64]. Overall, nondysplastic and low-grade cases harbored low frequency of LOH and MSI mutations while more advanced stages (HGD and OAC) shared an abundant ML profile.

This is consistent with a separate study where increased frequency of LOH correlated with severity of BO diagnosis [65]. The addition of MSI assessment in ten genomic loci to define ML, however, strengthened this correlation. The authors further suggest that in order to make MSI status a more reliable marker for genomic instability, the microsatellite markers, number of loci, and degree of aberration should be standardized [64].

In gastric cancer, MSI-high tumors are characterized by hypermutation, activation of mitotic pathways *MHL1* silencing are distinct from tumors harboring chromosomal instability. Further genomic characterization of adenocarcinomas arising at the gastroesophageal junction showed key features of chromosomal instability (TP53 mutation, A to C base changes, amplification of several genes in the RTK-RAS pathway and deletion of PTEN) while tumors originating in the body and antrum display high microsatellite instability [66].

Accumulation of Fragile Sites

Fragile Sites are local chromosomal regions prone to DNA breaks and rearrangements leading to genetic alterations such as amplifications, deletions, and translocations. One of the most well-characterized fragile sites, *FRA3B*, is contained within the Fragile Histidine Triad (*FHIT*), a ~1000 kb gene that encodes an enzyme involved in DNA damage response [67]. Subsequently, reduction of *FHIT* gene expression is associated with aberrant cell cycle checkpoint and dysregulated homologous recombination [68]. In mouse and in vitro models, *FHIT* overexpression induces apoptosis and reduces tumor growth [69]. One of the early studies to consider its involvement in tumor suppression analyzed 27 cases of upper and lower gastrointestinal carcinomas and showed that 50 % had aberrant *FHIT* transcripts [70]. It has been suggested that LOH and deletions of fragile regions are early indications of oncogenesis [71]. In a study of 20 patients with premalignant BO, the most frequent copy number losses were observed at *FRA3B/FHIT* (81 %) [72]. Additionally, aberrant *FHIT* transcripts were observed even in nonmalignant tissues [73]. Genome-wide analysis of copy number alterations in OAC using single nucleotide polymorphism (SNP) array showed that deletion of *FRA3B/FHIT* is the most common of all fragile sites [35, 74–76]. Evaluation by immunohistochemistry showed reduction of FHIT expression in 33 % of adenocarcinoma cases [77].

Another well-studied weak region of the genome, *FRA16D*, which overlaps with the double tryptophan (WW) domain-containing oxidoreductase (*WWOX*) gene. Activation of *WWOX* is an essential component of TNF signaling and, thus, involved in stress-induced apoptosis [78]. Recently, Abu-Odeh et al. characterized its mechanistic role in DNA damage response via interaction with the checkpoint kinase ataxia-telangiectasia-mutated (*ATM*). Consequently, reduction of *WWOX* expression led to *ATM* inactivation and impaired DNA repair. In mice, complete knockout of *WWOX* resulted to early tumor formation [79, 80]. Deregulation of this locus has been reported in several malignancies including esophageal cancer. Although previously more associated with the squamous

carcinoma type, recent copy number alteration (CNA) analysis in BO and OAC has shown that LOH and biallelic deletions within the *WWOX* locus occur at a frequency second only to FRA3B/FHIT loss [41, 72, 76, 81].

Dysfunctional Homologous Recombination and Telomere Biology

Telomere shortening is correlated with cellular senescence and apoptosis. Although continuous cell division leads to shortened chromosomal ends, cancer cells are able to escape apoptotic pathways by activating mechanisms involved in telomere elongation and stabilization, endowing them with unlimited replicative and proliferative potential. Telomerase is the enzyme responsible for telomere maintenance. In esophageal carcinogenesis, the expression of telomerase is increased as the disease progresses from metaplasia to adenocarcinoma [82]. Moreover, normal tissues from cancer patients showed significantly higher telomerase activity when compared with normal squamous samples from patients without cancer, suggesting that activation of the telomerase machinery is an early event in maintaining the genomic instability even in premalignant condition [83]. Results from a separate study further specified that the transition from low- to high-grade dysplasia showed the most substantial increase in telomerase expression [84]. Inhibition of telomerase leads to shortened telomeres, reduced cell growth, and apoptosis [82].

In normal cells, telomere maintenance is also achieved by homologous recombination (HR) [85, 86]. Truncated telomere in BO and OAC is also associated with genetic rearrangement [87] as unprotected and exposed chromosomal ends may undergo repetitive bridge-breakage-fusion cycle [57, 88–90]. Quantification via fluorescent in situ hybridization shows that telomere shortening is frequent in both premalignant and neoplastic tissues [87] even in the presence of increased telomerase activity [82]. Specifically, widespread telomere loss is more notable in chromosomes with frequent rearrangements. Seventy-seven percent of OAC cases ($N=22$) with evidence of oncogenic amplification through either chromothripsis-derived double-minute chromosome formation or breakage-fusion-bridge were observed to have shortened telomeric ends [57]. The recombination enzyme RAD51 is also upregulated and HR activity is elevated in OAC cell lines and tissue specimens [91]. Interestingly, telomerase inhibition alone led to further increase in RAD51 expression and subsequent HR activity. Combination of HR suppression with telomerase inhibition, however, reduced telomere length and increased the rate of apoptosis [92].

Epigenetic Alterations in BO and OAC

Epigenetic changes are non-DNA sequence modifications that can lead to altered gene expression without the occurrence mutation and/or structural variation. Over the past years, the transition from locus-specific studies to genomic multiplatform

approaches has enriched our understanding of the spectrum of epigenetic alterations in malignant progression of BO to OAC.

The hypermethylated status of tumor suppressor genes such as *APC, CDKN2A, CDH1,* and *REPRIMO* along with the transcription factor ESR1 has been defined in several studies [93]. As previously described, anomalies in *CDKN2A*, including silencing through CpG island methylation [94], are early events in aberrant esophageal tissue transformation [95]. Methylation of the promoter region of *APC* is also one of the most common epigenetic alterations in OAC [96]. *APC* promoter region methylation was observed to be as high as 95 % (48/52) in OAC and 39.5 % (17/34) in BO whereas no alteration was seen in match normal tissues. In this study by Kawakami et al. match plasma samples were also assayed for methylation patterns. The authors reported that high plasma levels of methylated *APC* DNA were detected in 13 out of 25 patients. Moreover, increased *APC* methylation correlated significantly with poor patient survival [97]. More comprehensive approaches include the evaluation of gene panels. Analysis of four CpG islands in 107 biopsies from BO with HGD ($N=2$) and OAC ($N=4$) patients revealed that hypermethylation of *APC, CDKN2A,* and *ESR1* occurs at high frequency [98]. *REPRIMO* is involved in the p53-mediated cell cycle arrest and has been reported to be heavily methylated in esophageal cancer [99]. In one study, the frequency of *REPRIMO* methylation was 36 % (9/25) in BO, 65 % (7/11) in HGD, and 63 % (47/75) in OAC whereas it only occurred in 13 % (6/45) OSCC cases and was not present in 19 normal epithelium samples. Based on these results, the authors suggested that *REPRIMO* methylation may be an early epigenetic alteration specific to columnar cells [99]. Other genes that have been reported to be silenced through hypermethylation in OAC are listed on Table 12.3.

An integrated study that incorporated copy number alteration via array comparative genomic hybridization (aCGH) and transcriptomic data with genome-wide methylation profiling, in contrast, reported that global hypomethylation with accompanying gene amplification is more frequent in various stages of progression to malignancy. Upregulated genes including *CXCL1, CXCL3, GATA6,* and *DMBT1* were suspected to be oncogenic drivers in BO. In addition, differential methylation was observed outside the canonical CpG islands and also included distant CG dinucleotides [100]. Hypomethylation of noncoding DNA regions has also been reported. Analysis of the methylation status of 1.8 million CpG sites through massive parallel sequencing of matched normal, BO and OAC samples revealed genome-wide hypomethylation in intragenic, repetitive elements and noncoding regions. More importantly, the resulting epigenetic profile of noncoding regions of dysplastic and tumor samples was distinctly different from the matched normal tissues [101].

In recent years, studies on the regulation of gene expression included regions of DNA actively transcribing small noncoding microRNA (miRNA) capable of degrading target mRNA via sequence complementarity. miR-21 is one of most abundantly expressed microRNAs in both OSCC and OAC. Using miroarray-based profiling and clustering analyses, one study reported a fivefold increase in tumor-normal comparison [102]. In a more recent two-step study, microarray data revealed 34 miRNAs differentially expressed in normal squamous epithelium ($N=11$) and BO/OAC

Table 12.3 Genes with differential methylation profile in esophageal adenocarcinoma

Gene	Role	Status	Cytoband	Reference
CDKN2A (p16)	Tumor suppressor	Hypermethylated	9p21.3	[33]
MGMT	DNA repair	Hypermethylated	10q26.3	
RUNX3	Transcription factor	Hypermethylated	1p36.11	
AKAP12	Scaffold protein	Hypermethylated	6q25.1	[116]
NELL1	Calcium ion binding	Hypermethylated	11p15.1	[116]
TAC1	Peptide hormone	Hypermethylated	7q21.3	[116]
SST	Peptide hormone	Hypermethylated	3q27.3	[116]
CDH13	Cell–cell adhesion	Hypermethylated	16q23.3	[116]
TMEFF2 (HPP1)	Tumor suppressor	Hypermethylated	2q32.3	[116]
SLC22A18	Tumor suppressor	Hypermethylated	11p15.4	[116]
PIGR	Fc receptor	Hypermethylated	1q32.1	[117]
GJA12	Gap junction protein	Hypermethylated	1q42.13	[117]
RIN2	GTPase/membrane transport	Hypermethylated	20p11.22	[117]
REPRIMO	Tumor suppressor	Hypermethylated	2q23.3	[99]
CXCL1	Chemokine ligand	Hypomethylated	4q13.3	[100]
CXCL3	Chemokine ligand	Hypomethylated	4q13.3	[100]
GATA-6	Transcription factor	Hypomethylated	18q11.2	[100]
DMBT1	Immune system mediator	Hypomethylated	10q26.13	[100]
CDX1	Transcription factor	Hypomethylated	5q32	[100]

($N=25$). Five other other miRNAs, including miR-21, were validated in a separate cohort ($N=18$) using quantitative reverse transcription polymerase reaction. Although miRNA profiling in this study differentiated normal squamous from columnar and dysplastic tissue, it did not distinguish BO from OAC samples [103]. Functional studies have also revealed the putative role of some of these miRNAs in esophageal carcinogenesis. For example, the downregulated miR-375 is associated with *MYC* and *TP53* regulation [101, 104, 105]. Other miRNAs that are significantly expressed with progression to malignancy include miR-192 [106], miR-194, and miR-96a [107] while some, such as miR-200 [108] and miR-203, are downregulated in BO and OAC cases when compared with normal esophageal mucosa [102]. The unique expression profiles of miRNAs in normal tissues and tumors at different stages of malignant progression makes them potential diagnostic indicators [109]. In a comprehensive review of miRNAs in esophageal carcinogenesis by Sakai et al. miR-25, -99a, -133a, and -133b were considered to have purely diagnostic potential while miR-21, -27b, -126, -143, and -145 as a panel may hold value in both diagnosis and prediction of progression [110]. Optimized clinical platforms for miRNA profiling in serum and formalin-fixed tissues may, therefore, become valuable tools in characterizing pathologic status. Optimized clinical platforms for miRNA profiling in serum and formalin-fixed tissues may, therefore, become valuable tools in characterizing pathologic status.

Biomarker Development

Comprehensive analysis of human tumors has uncovered wide-ranging information that can be applied in the clinic for enhanced screening methods and more reliable diagnostic practice. Unfortunately, the translation of many preclinical research findings is frequently not immediate due to the steps required for clinical implementation. In the case of OAC, the main challenge continues to be the identification of high-risk patients. In its broadest definition, a biomarker may include endoscopic/imaging findings, physiologic measurements, or molecular indicators [111]. To improve collaboration and facilitate efficiency, the National Cancer Institute Early Detection Research Network (EDRN) has launched a biomarker validation pipeline that covers phases from discovery to clinical implementation [112].

Because BO is the only known premalignant condition for OAC and not all patients diagnosed with BO will experience neoplastic progression, research studies have concentrated on biomarkers that (1) could identify potential progressors that could benefit from intensive surveillance and spare the patients with stable conditions from unnecessary and expensive medical procedures, and (2) diagnose carcinoma in its earliest stage with high sensitivity and specificity so that treatment can be implemented. In recent years, there has been an abundance of studies for different candidate biomarkers, and the number of prospective studies with robust sample-sizes is increasing. Some examples of different types of biomarker panels recently tested in studies with large, prospective cohorts are listed in Table 12.4.

p53

Because of its recognized role in maintaining the integrity of the genome, several studies have utilized p53 status for ascertaining cases at risk for progression to OAC [113]. In a case–control study, biopsies from 635 BO patients included in a larger prospective cohort were analyzed for p53 abnormalities using immunohistochemistry. Progression to malignancy was correlated with overexpression of p53, although the risk is increased almost threefold with complete loss of p53 (adjusted relative risks [RRa] 5.6; 95% CI 3.1–10.3, [RRa] 14.0; 95% CI 5.3–37.2, respectively). Furthermore, p53 aberration co-occurring with LGD had more than twice the predictive value for malignant progression compared to histological diagnosis of LGD alone (15% vs. 33%, respectively). The large prospective follow-up study by Davelaar et al. is the latest investigation of the reliability of TP53 as a biomarker. In this study, brush cytology samples from a total 116 patients ($N=80$ IM; $N=13$ IND; $N=7$ LGD; $N=6$ HGD; $N=10$ OAC) were analyzed using IHC and FISH for TP53 abnormalities. IHC staining detected 27 patients ($N=8$ IM; $N=7$ LGD; $N=4$ HGD; $N=8$ OAC) with *TP53* aberration while FISH analysis only identified 22 cases ($N=9$ IM; $N=2$ LGD; $N=4$ HGD; $N=7$ OAC). Combination of the two techniques resulted in detection of all patients ($N=40$) confirmed to harbor *TP53* abnormalities. In addition, the increased detection rate of both

Table 12.4 Examples of different types of Biomarker Panels tested in large prospective cohorts

Purpose	Panel(s)	Study type	Cohort	Findings	Reference
Diagnostic/ risk stratification	Methylation: *SLC22A18, PIGR, GJA12, and RIN2*	Retrospective	60 BO; 36 DBO; 90 OAC	Stratified patients into low risk: <2 genes, intermediate: 2, and high: >2 based on number of methylated genes	[117]
		Prospective	61 BE; 28 DBO; 9 OAC		
Diagnostic (HGD vs. OAC)	Amplification: *c-myc, EGFR*, and 20q12 loci	Prospective	99 Patients (BO & OAC)	In HGD, the levels of amplification for *c-myc*, 20q13, and *EGFR* were 18 %, 13 %, 11 %, respectively	
Prediction of progression	9p/17p LOH, Abnormal DNA content	Prospective	243 BO	Three-marker panel had better OAC risk prediction than any single marker (alone)	[119]
Prediction of progression	Obesity markers	Prospective	392 BO	High levels of leptin correlated with increased OAC risk (HR = 2.51; 95 % CI 1.09–5.81) within 3 years. The inverse is true for levels of adiponectin (HR = 0.34; 95 % CI, 0.14–0.82)	[125]
Prediction of progression	p53: IHC/FISH	Prospective	116 BO	Combination of IHC and FISH detected all patients with p53 abnormalities; 9 out of 11 patients confirmed to have aberrant p53 progressed to EAC	[114]

BO Barrett's esophagus, *DBO* dysplastic Barrett's esophagus, *OAC* esophageal adenocarcinoma, *HR* hazard ratio, *CI* confidence interval, *IHC* immunohistochemistry

FISH and IHC to identify *TP53* loss and positivity, respectively, correlated very well with the severity of the diagnosis. In order to explore the predictive value of *TP53* aberrations detected by IHC and FISH, the investigators performed a prospective follow-up of the cohort for 7 years. A total of 91 patients remained eligible. Twenty-one out of the 91 patients tested positive for TP53 abnormality. Of the 11

progressors, 9 had a TP53 abnormality and were thus correctly classified as at risk. When compared to single-method detection, FISH + IHC had a better sensitivity of (FISH + IHC:81.8; IHC: 63.6; FISH: 36.4) [114]. In the recent update of the British Society of Gastroenterology guidelines for the diagnosis and management of BO, p53 IHC was suggested to be included in routine assessment in order to augment histopathological diagnosis of dysplasia. This suggestion is supported by evidence from previous studies where inclusion of p53 analysis diminished diagnostic variability between pathologists [5].

Epigenetic Biomarkers

Cumulative studies on epigenetic profiling of BO and OAC by Meltzer and colleagues produced a panel of methylated genes consisting of *p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12*, and *CDH13* of progression to malignant neoplasm in BO. These methylation biomarkers have been validated in a multicentre double-blinded study consisting of 145 nonprogressors and 50 progressors. This study utilized a tiered risk stratification model previously described [115]. Although the panel and the model incorporated had a specificity of 0.9, its sensitivity for prediction of progression was only 50 % [116].

With the goal to establish a biomarker panel to risk stratify patients, Alvi et al. used methylation arrays to investigate genes that are able to discriminate 22 BO and 24 OAC samples. Using pyrosequencing, a unique gene panel consisting of *SLC22A18, PIGR, GJA12,* and *RIN2* with the best area under the curve (AUC) score (0.988), sensitivity (94 %), and specificity (97 %) was validated twice, first on a retrospective cohort ($N=60$ NDBO; $N=26$ dysplastic BO; $N=90$ OAC) then in a prospective multicenter study ($N=98$ with varying diagnosis). The results from the prospective validation showed that the probability of progressing to HGD/OAC is more likely as the number of methylation markers increases [117].

Inflammation and Stress Biomarkers

Because chronic exposure of esophageal epithelium leads to inflammation, oxidative stress, and subsequent development of BO, Hardikar et al. explored whether inflammation markers [C-reactive protein (CRP), interleukin-6 (IL6), soluble tumor necrosis factor (sTNF) receptors I and II] and oxidative stress indicators (F2-isoprostanes) predict progression to OAC. A total of 397 patients ($N=352$ BO; $N=45$ OAC) participated in this prospective study. Plasma samples were taken from the patients at two time points for the analysis of their levels of inflammation and oxidative stress markers. The authors found that above the median concentrations of CRP increased the probability of progression by 80 % and that patients with abundant IL6 had a twofold increased risk of progression while levels of TNF receptors and F2-isoprostanes were not associated with progression to OAC [118].

Chromosomal Abnormality Panel

Genetic content abnormalities (tetraploidy and aneuploidy) occur as a result of the loss of integrity of the genome and are suggested to occur after the accumulation of *CDKN2A* and *TP53* aberrations [52]. Analysis of biopsy samples by flow cytometry revealed patients with aneuploidy and tetraploidy had five-yr OAC incidences of 43 and 56 %, respectively. In this prospective study, all patients who progressed to OAC within 5 years had baseline aneuploidy and tetraploidy [28]. In another prospective study of 243 patients with BO, Galipeau et al. used a panel of somatic genetic abnormalities (SGAs), which included *TP53* and *CDKN2A* (p16) LOH, tetraploidy and aneuploidy (risk ratio [RR] = 38.7; 95 % CI 10.8–138.5, $P < 0.001$), to investigate the effect of nonsteroidal inflammatory drugs in BO progression. Patients without initial genetic abnormality had a 12 % 10-year cumulative OAC risk while those with 19p/17p LOH and genetic abnormalities had ~79 % 10-year OAC incidence. NSAIDS significantly reduced the risk of progression especially in those with multiple genetic alterations (NSAID nonusers 79 % 10-year OAC risk; NSAID users: 30 %; $P < 0.001$) [119]. In a related follow-up prospective study ($N = 13$ BO patients; median follow-up surveillance 11.6 years), the authors investigated the dynamics of SGAs over time and provided evidence that NSAIDs decrease the rate at which SGAs accumulate [120].

In a population-based, nested case–control study of BO patients ($N = 380$) in Northern Ireland, abnormal DNA content, low-grade dysplasia, and Aspergillus oryzae lectin (AOL) reliably discriminated progressors ($N = 89$) from nonprogressors ($N = 291$). In this study, patients were more likely to progress to cancer if they had LGD and co-occurring factor(s). Specifically, the odds ratio (OR) for neoplastic progression for LGD patients was 3.74 for every biomarker in contrast to an OR of 2.99 in those patients without dysplasia [121].

Imaging with Molecular Biomarkers

Because endoscopic surveillance is prone to sampling bias and histological visual scoring continues to be an inherently subjective approach, di Pietro et al. investigated whether autofluorescence imaging (AFI) could be used to help the endoscopist to target biopsies, which could then be graded more objectively. Thus, the idea was to combine an imaging method with a molecular biomarker. First, the investigators performed a cross-sectional prospective study consisting of 157 patients. The multiplatform biomarker panel included: aneuploidy/tetraploidy; 9p and 17p loss of heterozygosity; *RUNX3, HPP1,* and *CDKN2A* methylation; p53 and cyclin A immunohistochemistry. Bootstrap resampling was used to select the best diagnostic biomarker panel for HGD and early cancer (EC). This panel was validated in an independent cohort of 46 patients. Aneuploidy, p53 immunohistochemistry, and cyclin A had the strongest association with dysplasia in the per-biopsy analysis and, as a panel, had an area under the

receiver operating characteristic curve of 0.97 (95% CI 0.95–0.99) for diagnosing HGD/OAC. The diagnostic accuracy for HGD/OAC of the three-biomarker panel from AFI+ areas was superior to AFI- areas ($P<0.001$). Compared with the standard protocol, this panel had equal sensitivity for HGD/EC, with a 4.5-fold reduction in the number of biopsies. In an independent validation cohort of patients, the panel had a sensitivity and specificity for HGD/EC of 100 and 85%, respectively [122].

Significance of Epidemiological Risk Factors

Apart from gastroesophageal reflux disease (GORD), risk factors for the development of BO include body mass index (BMI,) central obesity, age over 50 years old, male gender, white race, and presence of hiatal hernia [123]. In a prospective study reported recently ($N=24,068$), Yates et al. found that participants with high BMI are more likely to develop BO and progress to OAC [124]. In a separate study, high levels of leptin (HR, 2.51; 95% CI, 1.09–5.81; P trend=0.03 within 3 years; HR, 2.07; 95% CI, 1.01–4.26; P trend=0.048 within 6 years), insulin resistance (HR, 2.45; 95% CI, 1.43–4.1; P trend=0.001), and low levels of adiponectin (HR, 0.34; 95% CI, 0.14–0.82) were associated with increased risk for OAC [125]. Other epidemiological factors that have been recently explored include tea and coffee consumption, smoking, alcohol consumption and fruit, vegetable, and nitrate intake [126]. In future studies it will be important to combine epidemiological risk factors with molecular biomarkers.

Molecular Alternatives to Endoscopy

There has been significant research in the development of cost-effective and minimally invasive procedures to acquire and analyze tissue samples that can then be used for molecular analysis. The Cytosponge is a noninvasive cell-collecting device contained in a capsule that is swallowed and retrieved along with the cells collected in the lining of the esophagus. The specimens are then analyzed for expression of Trefoil Factor 3 (TFF3), a marker of columnar epithelium evaluated in a previous gene expression study intended to discriminate BO from other tissues of the upper gastrointestinal area and the oropharynx [127]. In its most recent evaluation that involved 1,110 participants ($N=463$ controls with dyspepsia and reflux symptoms and $N=647$ BO), it was preferred by patients over conventional endoscopy when measured on a visual analog scale. Its overall specificity was 92.4% while its sensitivity increased from 79.9 to 87.2% with increasing BO segment [128]. In a separate study, samples collected from this device have also been analyzed for *TP53* aberrations in order to discriminate patients with varying grades of dysplastic BO. Collected samples from control patients or NDBO showed no *TP53* mutations while 86% (19/22) of HGD patients were detected to have *TP53* mutations [18] (Fig. 12.1).

Fig. 12.1 The cytosponge cell sampling device comes in a pill-like container, which dissolves in the stomach after swallowing. The sponge is then retrieved by pulling on the string collecting cells on its return passage. Combined cytology and immunohistochemistry allows the researcher to detect TFF3-positive goblet cells indicative of BE

Serum Markers

The tissue-specific gene regulation of miRNAs makes them a good potential biomarker that can be sampled from noninvasive blood collection. In a pilot study, Bansal et al. selected a three-miRNA panel (miR-192-5p, miR-195-5p, and miR-215-5p) based on their differential expression in GORD and BO for further testing in a discovery cohort ($N=40$ GORD; $N=27$ BO) and validation in an independent cohort ($N=19$ GORD; $N=11$ BO). The diagnostic accuracy of the miRNA panel was calculated using receiver operating curves (ROC) and, for specificity, the gastric cardia epithelium and the nonintestinal columnar epithelium was used for comparison. The reported AUC, sensitivity, and specificity for the three-miRNA panel were 0.96–0.97, 92–100, and 94–95 %, respectively, in the discovery cohort. These measures were relatively similar in the validation cohort where the sensitivity and specificity of the miRNAs for diagnosing BO were 92 and 94 %, AUC 0.94 (0.80–0.99, $P=0.0004$), respectively, for miR-192-5p while miR-215-5p had 100 and 94 %, AUC 0.98 (0.84–1, $P=0.0004$); and miR-194-5p, 91 and 94 %, AUC 0.96 (0.80–0.99, $P=0.0001$), respectively. In both cohorts, the panel successfully recognized samples with columnar background. Also, after comparison with mRNA profiles of gastric and intestinal columnar epithelia, the authors found that miR-194-5p and miR-215-5p successfully discriminated BO patients with intestinal-type columnar epithelium [129].

Double cortin-like kinase 1 (DCLK1) is a candidate stem cell marker involved in the expression of transcription factors inducing epithelial–mesenchymal transition in other cancers. It is found to be overexpressed in BO and OAC [130]. Whorton et al. prospectively investigated whether the immunohistochemical expression of DCLK1 was associated with detectable DCLK1 plasma expression in patients with existing BO and OAC. To explore this, biopsy samples from 40 patients ($N=10$

normal controls, $N=13$ NDBO, $N=9$ dysplastic BO, and $N=8$ OAC) were stained using DCLK1 antibody. Plasma samples were collected as well and subjected to Western Blot and ELISA analysis. While the results showed that increased expression of DCLK1 in the BO/OAC tissues correlated with the abundant presence of DCLK1 in the plasma [131], it did not discriminate patients with different grades of BO, which makes it unlikely to risk stratify patients with premalignant conditions.

In another study, the glycoprotein and putative stem cell marker CD133 was combined with p504s and Twist. CD133 and p504s have been evaluated individually in several studies. p504s (aplha-methylacyl-coenzyme A racemase) is an enzyme involved in beta-oxidation of branched chains fatty acids and bile acid intermediates [132]. Ahmad et al. investigated the differential expression of this panel in a cross-sectional study ($N=25$ BO; $N=25$ LGD; $N=25$ OAC; $N=25$ esophagectomy resections from OSCC patients) to evaluate their potential as biomarkers of malignant progression. The immunohistochemical analysis showed that p504s and CD133, but not Twist, had modest sensitivity in distinguishing dysplastic BO and OAC from nondysplastic BO, respectively, as evidenced by their overexpression [132].

Conclusion

Validation of biomarkers requires thorough preclinical studies establishing reproducibility of results and its specificity and sensitivity. Furthermore, biomarkers need to address the issues of under- and overdiagnosis, particularly during premalignant conditions such as BO. These issues are currently being addressed by large collaborative prospective trials that involve the participation of a substantial number of patients willing to undergo routine and/or specialized assessments over a significant span of time. Although methods incorporating genome-wide analysis have great potential to uncover the process of neoplastic progression to OAC, providing windows for detection and intervention, simplification of multiplatform genomic approaches to routine clinical techniques might pose a challenge. At present, objective measurements of mutations, methylation patterns, protein and miRNA profiles, and molecular imaging are not yet feasible. To achieve clinical applicability, biomarker assessment requires an integrated yet simplified approach. Standardizing protocols can lead to tailored screening practices that could identify high-risk patients. So far, methylation panels and p53 immunostaining have produced promising results from prospective studies. Continued efforts should focus on the reproducibility of these results in studies with larger cohorts prior to clinical application. Lastly, collaborations, such as the Cancer Genome Atlas and International Cancer Genome Consortium, which further characterize the esophageal cancer genome greatly facilitate collaboration and exploration of potential diagnostic and predictive markers.

References

1. Montgomery EA, et al. Oesophageal cancer. In: Stewart BW, Wild CP. World Cancer Report 2014. World Health Organization; 2014. p. 528–543.
2. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013.
3. Cancer Research UK (CRUK) Oesophageal cancer. http://www.cancerresearchuk.org/cancer-help/type/oesophageal-cancer/. Accessed 31 Jan 2014.
4. Spechler SJ. Barrett esophagus and risk of esophageal cancer: a clinical review. JAMA. 2013;310:627–36.
5. di Pietro M, Fitzgerald RC. Screening and risk stratification for Barrett's esophagus: how to limit the clinical impact of the increasing incidence of esophageal adenocarcinoma. Gastroenterol Clin North Am. 2013;42(1):155–73. doi:10.1016/j.gtc.2012.11.006. Review.
6. Ali HR, Rueda OM, Chin SF, Curtis C, et al. Genome-driven integrated classification of breast cancer validated in over 7,500 samples. Genome Biol. 2014;15(8):431. doi:10.1186/s13059-014-0431-1.
7. Paul MK, Mukhopadhyay AK. Tyrosine kinase—role and significance in cancer. Int J Med Sci. 2004;1(2):101–15.
8. Ekman S, Bergqvist M, Heldin CH, Lennartsson J. Activation of growth factor receptors in esophageal cancer—implications for therapy. Oncologist. 2007;12:1165–77. doi:10.1634/theoncologist.12-10-1165.
9. Kwak EL, Jankowski J, Thayer SP, Lauwers GY, Brannigan BW, Harris PL, Okimoto RA, Haserlat SM, Driscoll DR, Ferry D, Muir B, Settleman J, Fuchs CS, Kulke MH, Ryan DP, Clark JW, Sgroi DC, Haber DA, Bell DW. Epidermal growth factor receptor kinase domain mutations in esophageal and pancreatic adenocarcinomas. Clin Cancer Res. 2006;12(14 Pt 1):4283–7.
10. Gowryshankar A, Nagaraja V, Eslick GD. HER2 status in Barrett's esophagus and esophageal cancer: a meta analysis. J Gastrointest Oncol. 2014;5(1):25–35. doi:10.3978/j.issn.2078-6891.2013.039.
11. Langer R, Rauser S, Feith M, Nährig JM, Feuchtinger A, Friess H, Höfler H, Walch A. Assessment of ErbB2 (Her2) in oesophageal adenocarcinomas: summary of a revised immunohistochemical evaluation system, bright field double in situ hybridisation and fluorescence in situ hybridisation. Mod Pathol. 2011;24(7):908–16. doi:10.1038/modpathol.2011.52.
12. Wei Q, Chen L, Sheng L, Nordgren H, Wester K, Carlsson J. EGFR, HER2 and HER3 expression in esophageal primary tumours and corresponding metastases. Int J Oncol. 2007;31(3):493–9.
13. Kim J, Fox C, Peng S, Pusung M, Pectasides E, Matthee E, Hong YS, Do IG, Jang J, Thorner AR, Van Hummelen P, Rustgi AK, Wong KK, Zhou Z, Tang P, Kim KM, Lee J, Bass AJ. Preexisting oncogenic events impact trastuzumab sensitivity in ERBB2-amplified gastro-esophageal adenocarcinoma. J Clin Invest. 2014;124(12):5145–58. doi:10.1172/JCI75200.
14. Torquati A, O'rear L, Longobardi L, Spagnoli A, Richards WO, Daniel Beauchamp R. RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells. Surgery. 2004;136(2):310–6.
15. Garrigue-Antar L, Souza RF, Vellucci VF, Meltzer SJ, Reiss M. Loss of transforming growth factor-beta type II receptor gene expression in primary human esophageal cancer. Lab Invest. 1996;75(2):263–72.
16. Boonstra JJ, van Marion R, Douben HJ, Lanchbury JS, Timms KM, et al. Mapping of homozygous deletions in verified esophageal adenocarcinoma cell lines and xenografts. Genes Chromosomes Cancer. 2012;51(3):272–82.

17. Dulak AM, Stojanov P, Peng S, Lawrence MS, Fox C, et al. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. Nat Genet. 2013;45(5):478–86.
18. Weaver JM, Ross-Innes CS, Shannon N, Lynch AG, Forshew T, et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet. 2014;46(8):837–43.
19. Singhi AD, Foxwell TJ, Nason K, Cressman KL, McGrath KM, et al. Smad4 loss in esophageal adenocarcinoma is associated with an increased propensity for disease recurrence and poor survival. Am J Surg Pathol. 2015;39(4):487–95.
20. Wang Y, Qin X, Wu J, Qi B, Tao Y, et al. Association of promoter methylation of RUNX3 gene with the development of esophageal cancer: a meta analysis. PLoS One. 2014;9(9):e107598.
21. Miller CT, Moy JR, Lin L, Schipper M, Normolle D, et al. Gene amplification in esophageal adenocarcinomas and Barrett's with high-grade dysplasia. Clin Cancer Res. 2003;9(13):4819–25.
22. Sarbia M, Arjumand J, Wolter M, et al. Frequent c-myc amplification in high-grade dysplasia and adenocarcinoma in Barrett esophagus. Am J Clin Pathol. 2001;115(6):835–40.
23. von Rahden BH, Stein HJ, Pühringer-Oppermann F, Sarbia M. c-myc amplification is frequent in esophageal adenocarcinoma and correlated with the upregulation of VEGF-A expression. Neoplasia. 2006;8(9):702–7.
24. Lagorce C, Paraf F, Vidaud D, Couvelard A, Wendum D, et al. Cyclooxygenase-2 is expressed frequently and early in Barrett's oesophagus and associated adenocarcinoma. Histopathology. 2003;42(5):457–65.
25. Prins MJ, Verhage RJ, ten Kate FJ, van Hillegersberg R. Cyclooxygenase isoenzyme-2 and vascular endothelial growth factor are associated with poor prognosis in esophageal adenocarcinoma. J Gastrointest Surg. 2012;16(5):956–66. doi:10.1007/s11605-011-1814-1.
26. Surget S, Khoury MP, Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. Onco Targets Ther. 2013;7:57–68. doi:10.2147/OTT.S53876.
27. Dolan K, Walker SJ, Gosney J, Field JK, Sutton R. TP53 mutations in malignant and premalignant Barrett's esophagus. Dis Esophagus. 2003;16(2):83–9.
28. Reid BJ, Prevo LJ, Galipeau PC, Sanchez CA, Longton G, Levine DS, Blount PL, Rabinovitch PS. Predictors of progression in Barrett's esophagus II: baseline 17p (p53) loss of heterozygosity identifies a patient subset at increased risk for neoplastic progression. Am J Gastroenterol. 2001;96(10):2839–48.
29. Chung SM, Kao J, Hyjek E, Chen YT. p53 in esophageal adenocarcinoma: a critical reassessment of mutation frequency and identification of 72Arg as the dominant allele. Int J Oncol. 2007;31:1351–5.
30. Galipeau PC, et al. Clonal expansion and loss of heterozygosity at chromosomes 9p and 17p in premalignant esophageal (Barrett's) tissue. J Natl Cancer Inst. 1999;91:2087–95.
31. Maley CC, Galipeau PC, Li X, Sanchez CA, Paulson TG, Reid BJ. Selectively advantageous mutations and hitchhikers in neoplasms: p16 lesions are selected in Barrett's esophagus. Cancer Res. 2004;64(10):3414–27.
32. Hardie LJ, et al. p16 expression in Barrett's esophagus and esophageal adenocarcinoma: association with genetic and epigenetic alterations. Cancer Lett. 2005;217:221–30.
33. Bian YS, Osterheld MC, Fontolliet C, Bosman FT, Benhattar J. p16 inactivation by methylation of the CDKN2A promoter occurs early during neoplastic progression in Barrett's esophagus. Gastroenterology. 2002;122(4):1113–21.
34. Huang Y, Peters CJ, Fitzgerald RC, Gjerset RA. Progressive silencing of p14ARF in oesophageal adenocarcinoma. J Cell Mol Med. 2009;13:398–409.
35. Dulak AM, Schumacher SE, van Lieshout J, Imamura Y, Fox C, et al. Gastrointestinal adenocarcinomas of the esophagus, stomach, and colon exhibit distinct patterns of genome instability and oncogenesis. Cancer Res. 2012;72(17):4383–93.
36. Chow AY. Cell cycle control by oncogenes and tumor suppressors: driving the transformation of normal cells into cancerous cells. Nat Educ. 2010;3(9):7.

37. Geddert H, Heep HJ, Gabbert HE, et al. Expression of cyclin B1 in the metaplasia—dysplasia—carcinoma sequence of Barrett esophagus. Cancer. 2002;94:212–8.
38. Arber N, Gammon MD, Hibshoosh H, et al. Overexpression of cyclin D1 occurs in both squamous carcinomas and adenocarci- nomas of the esophagus and in adenocarcinomas of the stomach. Hum Pathol. 1999;30:1087–92.
39. Morgan RJ, Newcomb PV, Hardwick RH, Alderson D. Amplification of cyclin D1 and MDM-2 in oesophageal carcinoma. Eur J Surg Oncol. 1999;25(4):364–7.
40. Sarbia M, Stahl M, Fink U, Heep H, Dutkowski P, Willers R, Seeber S, Gabbert HE. Prognostic significance of cyclin D1 in esophageal squamous cell carcinoma patients treated with surgery alone or combined therapy modalities. Int J Cancer. 1999;84(1):86–91.
41. Gu J, Ajani JA, Hawk ET, Ye Y, Lee JH, Bhutani MS, et al. Genomewide catalogue of chromosomal aberrations in Barrett's esophagus and esophageal adenocarcinoma: a high-density single nucleotide polymorphism array analysis. Cancer Prev Res (Phila). 2010;3:1176–86.
42. Semb H, Christofori G. The tumor-suppressor function of E-cadherin. Am J Hum Genet. 1998;63(6):1588–93. doi:10.1086/302173.
43. Falkenback D, Nilbert M, Oberg S, Johansson J. Prognostic value of cell adhesion in esophageal adenocarcinomas. Dis Esophagus. 2008;21(2):97–102. doi:10.1111/j.1442-2050.2007.00749.x.
44. Bongiorno PF, Al-Kasspooles M, Lee SW, Rachwal WJ, Moore JH, Whyte RI, Orringer MB, Beer DG. E-cadherin expression in primary and metastatic thoracic neoplasms and in Barrett's oesophagus. Br J Cancer. 1995;71(1):166–72.
45. Krishnadath KK, Tilanus HW, van Blankenstein M, Hop WC, Kremers ED, Dinjens WN, Bosman FT. Reduced expression of the cadherin-catenin complex in oesophageal adenocarcinoma correlates with poor prognosis. J Pathol. 1997;182(3):331–8.
46. Schauer MC, Stoecklein NH, Theisen J, Kröpil F, Baldus S, Hoelscher A, Feith M, Bölke E, Matuschek C, Budach W, Knoefel WT. The simultaneous expression of both ephrin B3 receptor and E-cadherin in Barrett`s adenocarcinoma is associated with favorable clinical staging. Eur J Med Res. 2012;17:10. doi:10.1186/2047-783X-17-10.
47. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev. 2003;17(14):1709–13.
48. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia. 2010;15(2):117–34. doi:10.1007/s10911-010-9178-9. Epub 2010 May 19.
49. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17(1):9–26. doi:10.1016/j.devcel.2009.06.016.
50. Moyes LH, McEwan H, Radulescu S, Pawlikowski J, Lamm CG, Nixon C, Sansom OJ, Going JJ, Fullarton GM, Adams PD. Activation of Wnt signalling promotes development of dysplasia in Barrett's oesophagus. J Pathol. 2012;228(1):99–112. doi:10.1002/path.4058.
51. Clément G, Braunschweig R, Pasquier N, Bosman FT, Benhattar J. Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. Oncogene. 2006;25(21):3084–92.
52. Galipeau PC, Cowan DS, Sanchez CA, et al. 17p (p53) allelic losses, 4N (G2/tetraploid) populations and progression to aneuploidy in Barrett's oesophagus. Proc Natl Acad Sci U S A. 1996;93:7081–4.
53. Reid BJ, Haggit RC, Rubin CE, Rabinovitch PS. Barrett's esophagus. Correlation between flow cytometry and histology in detection of patients at risk for adenocarcinoma. Gastroenterology. 1987;93(1):1–11.
54. Flejou JF, Doublet B, Potet F, Metayer J, Hemet J. DNA ploidy in adenocarcinoma of Barrett's esophagus. Ann Pathol. 1990;10:161–5.
55. Yu C, Zhang X, Huang Q, Klein M, Goyal RK. High-fidelity DNA histograms in neoplastic progression in Barrett's esophagus. Lab Invest. 2007;87(5):466–72.
56. Zhang X, Huang Q, Goyal RK, Odze RD. DNA ploidy abnormalities in basal and superficial regions of the crypts in Barrett's esophagus and associated neoplastic lesions. Am J Surg Pathol. 2008;32(9):1327–35.

57. Nones K, Waddell N, Wayte N, Patch AM, Bailey P, Newell F, Holmes O, Fink JL, Quinn MC, Tang YH, Lampe G, Quek K, Loffler KA, Manning S, Idrisoglu S, Miller D, Xu Q, Waddell N, Wilson PJ, Bruxner TJ, Christ AN, Harliwong I, Nourse C, Nourbakhsh E, Anderson M, Kazakoff S, Leonard C, Wood S, Simpson PT, Reid LE, Krause L, Hussey DJ, Watson DI, Lord RV, Nancarrow D, Phillips WA, Gotley D, Smithers BM, Whiteman DC, Hayward NK, Campbell PJ, Pearson JV, Grimmond SM, Barbour AP. Genomic catastrophes frequently arise in esophageal adenocarcinoma and drive tumorigenesis. Nat Commun. 2014;5:5224. doi:10.1038/ncomms6224.
58. Turnpenny P, Ellard S. Emery's elements of medical genetics. 12th ed. London: Elsevier; 2005.
59. Peltomäki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol. 2003;21(6):1174–9.
60. Koppert LB, Wijnhoven BP, van Dekken H, et al. The molecular biology of esophageal adenocarcinoma. J Surg Oncol. 2005;92:169–90.
61. Evans SC, Gillis A, Geldenhuys L, et al. Microsatellite instability in esophageal adenocarcinoma. Cancer Lett. 2004;212:241–51.
62. Meltzer SJ, Yin J, Manin B, Rhyu MG, Cottrell J, Hudson E, Redd JL, Krasna MJ, Abraham JM, Reid BJ. Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. Cancer Res. 1994;54:3379–82.
63. Gleeson CM, Sloan JM, McGuigan JA, Ritchie AJ, Weber JL, Russell SHE. Ubiquitous somatic alterations at microsatellite alleles occur infrequently in Barrett's-associated esophageal adenocarcinomas. Cancer Res. 1996;56:259–63.
64. Khara HS, Jackson SA, Nair S, Deftereos G, Patel S, Silverman JF, Ellsworth E, Sumner C, Corcoran B, Smith Jr DM, Finkelstein S, Gross SA. Assessment of mutational load in biopsy tissue provides additional information about genomic instability to histological classifications of Barrett's esophagus. J Gastrointest Cancer. 2014;45(2):137–45. doi:10.1007/s12029-013-9570-y.
65. Ellsworth E, Jackson SA, Thakkar SJ, Smith Jr DM, Finkelstein S. Correlation of the presence and extent of loss of heterozygosity mutations with histological classifications of Barrett's esophagus. BMC Gastroenterol. 2012;12(1):181. doi:10.1186/1471-230X-12-181.
66. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202–9. doi:10.1038/nature13480.
67. Saldivar JC, Shibata H, Huebner K. Pathology and biology associated with the fragile FHIT gene and gene product. J Cell Biochem. 2010;109(5):858–65.
68. Hu B, Han SY, Wang X, Ottey M, Potoczek MB, Dicker A, Huebner K, Wang Y. Involvement of the Fhit gene in the ionizing radiation-activated ATR/CHK1 pathway. J Cell Physiol. 2005;202(2):518–23.
69. Ishii H, Mimori K, Ishikawa K, Okumura H, Pichiorri F, Druck T, Inoue H, Vecchione A, Saito T, Mori M, Huebner K. Fhit-deficient hematopoietic stem cells survive hydroquinone exposure carrying precancerous changes. Cancer Res. 2008;68(10):3662–70. doi:10.1158/0008-5472.CAN-07-5687.
70. Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K, The FHIT. Gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell. 1996;84(4):587–97.
71. Mori M, Mimori K, Shiraishi T, Alder H, Inoue H, Tanaka Y, Sugimachi K, Huebner K, Croce CM. Altered expression of Fhit in carcinoma and precarcinomatous lesions of the esophagus. Cancer Res. 2000;60(5):1177–82.
72. Lai LA, Kostadinov R, Barrett MT, Peiffer DA, Pokholok D, Odze R, Sanchez CA, Maley CC, Reid BJ, Gunderson KL, Rabinovitch PS. Deletion at fragile sites is a common and early event in Barrett's esophagus. Mol Cancer Res. 2010;8(8):1084–94. doi:10.1158/1541-7786.MCR-09-0529.

73. Chen YJ, Chen PH, Lee MD, Chang JG. Aberrant FHIT transcripts in cancerous and corresponding non-cancerous lesions of the digestive tract. Int J Cancer. 1997;72(6):955–8.
74. Wiech T, Nikolopoulos E, Weis R, Langer R, Bartholomé K, Timmer J, Walch AK, Höfler H, Werner M. Genome-wide analysis of genetic alterations in Barrett's adenocarcinoma using single nucleotide polymorphism arrays. Lab Invest. 2009;89(4):385–97. doi:10.1038/labinvest.2008.67.
75. Frankel A, Armour N, Nancarrow D, Krause L, Hayward N, Lampe G, Smithers BM, Barbour A. Genome-wide analysis of esophageal adenocarcinoma yields specific copy number aberrations that correlate with prognosis. Genes Chromosomes Cancer. 2014;53(4):324–38. doi:10.1002/gcc.22143.
76. Davison JM, Yee M, Krill-Burger JM, Lyons-Weiler MA, Kelly LA, Sciulli CM, Nason KS, Luketich JD, Michalopoulos GK, LaFramboise WA. The degree of segmental aneuploidy measured by total copy number abnormalities predicts survival and recurrence in superficial gastroesophageal adenocarcinoma. PLoS One. 2014;9(1):e79079. doi:10.1371/journal.pone.0079079.
77. Chava S, Mohan V, Shetty PJ, Manolla ML, Vaidya S, Khan IA, Waseem GL, Boddala P, Ahuja YR, Hasan Q. Immunohistochemical evaluation of p53, FHIT, and IGF2 gene expression in esophageal cancer. Dis Esophagus. 2012;25(1):81–7. doi:10.1111/j.1442-2050.2011.01213.x.
78. Chen S-J, Huang S-S, Chang N-S. Role of WWOX and NF-kB in lung cancer progression. Transl Respir Med. 2013;1(1):15.
79. Aqeilan RI, Hagan JP, de Bruin A, Rawahneh M, Salah Z, Gaudio E, Siddiqui H, Volinia S, Alder H, Lian JB, Stein GS, Croce CM. Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. Endocrinology. 2009;150(3):1530–5. doi:10.1210/en.2008-1087.
80. Abu-Odeh M, Bar-Mag T, Huang H, Kim T, Salah Z, Abdeen SK, Sudol M, Reichmann D, Sidhu S, Kim PM, Aqeilan RI. Characterizing WW domain interactions of tumor suppressor WWOX reveals its association with multiprotein networks. J Biol Chem. 2014;289(13):8865–80. doi:10.1074/jbc.M113.506790.
81. Nancarrow DJ, Handoko HY, Smithers BM, Gotley DC, Drew PA, Watson DI, Clouston AD, Hayward NK, Whiteman DC. Genome-wide copy number analysis in esophageal adenocarcinoma using high-density single-nucleotide polymorphism arrays. Cancer Res. 2008;68(11):4163–72. doi:10.1158/0008-5472.CAN-07-6710.
82. Shammas MA, Koley H, Beer DG, Li C, Goyal RK, Munshi NC. Growth arrest, apoptosis, and telomere shortening of Barrett's-associated adenocarcinoma cells by a telomerase inhibitor. Gastroenterology. 2004;126(5):1337–46.
83. Lord RV, Salonga D, Danenberg KD, Peters JH, DeMeester TR, Park JM, Johansson J, Skinner KA, Chandrasoma P, DeMeester SR, Bremner CG, Tsai PI, Danenberg PV. Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, adenocarcinoma sequence. J Gastrointest Surg. 2000;4(2):135–42.
84. Morales CP, Lee EL, Shay JW. In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer. 1998;83(4):652–9.
85. Cerone MA, Londono-Vallejo JA, Bacchetti S. Telomere maintenance by telomerase and by recombination can coexist in human cells. Hum Mol Genet. 2001;10(18):1945–52.
86. Verdun RE, Karlseder J. The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. Cell. 2006;127(4):709–20.
87. Finley JC, Reid BJ, Odze RD, Sanchez CA, Galipeau P, Li X, Self SG, Gollahon KA, Blount PL, Rabinovitch PS. Chromosomal instability in Barrett's esophagus is related to telomere shortening. Cancer Epidemiol Biomarkers Prev. 2006;15(8):1451–7.
88. Blackburn EH. Structure and function of telomeres. Nature. 1991;350(6319):569–73.
89. Mathieu N, Pirzio L, Freulet-Marrière MA, Desmaze C, Sabatier L. Telomeres and chromosomal instability. Cell Mol Life Sci. 2004;61(6):641–56.
90. Rodier F, Kim SH, Nijjar T, Yaswen P, Campisi J. Cancer and aging: the importance of telomeres in genome maintenance. Int J Biochem Cell Biol. 2005;37(5):977–90.

91. Pal J, Bertheau R, Buon L, Qazi A, Batchu RB, Bandyopadhyay S, Ali-Fehmi R, Beer DG, Weaver DW, Shmookler Reis RJ, Goyal RK, Huang Q, Munshi NC, Shammas MA. Genomic evolution in Barrett's adenocarcinoma cells: critical roles of elevated hsRAD51, homologous recombination and Alu sequences in the genome. Oncogene. 2011;30(33):3585–98. doi:10.1038/onc.2011.83.
92. Lu R, Pal J, Buon L, Nanjappa P, Shi J, Fulciniti M, Tai YT, Guo L, Yu M, Gryaznov S, Munshi NC, Shammas MA. Targeting homologous recombination and telomerase in Barrett's adenocarcinoma: impact on telomere maintenance, genomic instability and tumor growth. Oncogene. 2014;33(12):1495–505. doi:10.1038/onc.2013.103.
93. Monkhouse SJW, Muhlschlegel J, Barr H. Biomarkers in esophageal adenocarcinoma. Cancer biomarkers: minimal and noninvasive early diagnosis and prognosis. 2014. p. 321.
94. Wong DJ, Barrett MT, Stöger R, Emond MJ, Reid BJ. p16INK4a promoter is hypermethylated at a high frequency in esophageal adenocarcinomas. Cancer Res. 1997;57:2619–22.
95. Paulson TG, Galipeau PC, Xu L, Kissel HD, Li X, et al. p16 mutation spectrum in the premalignant condition Barrett's esophagus. PLoS One. 2008;3(11):e3809. PubMed PMID: 19043591, PubMed Central PMCID: PMC2585012.
96. Agarwal A, Polineni R, Hussein Z, Vigoda I, Bhagat TD, Bhattacharyya S, Maitra A, Verma A. Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. Int J Clin Exp Pathol. 2012;5(5):382–96.
97. Kawakami K, Brabender J, Lord RV, Groshen S, Greenwald BD, Krasna MJ, Yin J, Fleisher AS, Abraham JM, Beer DG, Sidransky D, Huss HT, Demeester TR, Eads C, Laird PW, Ilson DH, Kelsen DP, Harpole D, Moore MB, Danenberg KD, Danenberg PV, Meltzer SJ. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J Natl Cancer Inst. 2000;92(22):1805–11.
98. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PW, Skinner KA. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res. 2000;60(18):5021–6.
99. Hamilton JP, Sato F, Jin Z, Greenwald BD, Ito T, Mori Y, Paun BC, Kan T, Cheng Y, Wang S, Yang J, Abraham JM, Meltzer SJ. Reprimo methylation is a potential biomarker of Barrett's-associated esophageal neoplastic progression. Clin Cancer Res. 2006;12(22):6637–42.
100. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari MJ, Yu Y, Montagna C, Montgomery EA, Canto M, Dunbar KB, Wang J, Roa JC, Mo Y, Bhagat T, Ramesh KH, Cannizzaro L, Mollenhauer J, Thompson RF, Suzuki M, Meltzer SJ, Melnick A, Greally JM, Maitra A, Verma A. Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. PLoS Genet. 2011;7(3):e1001356. doi: 10.1371/journal.pgen.1001356. Epub 2011 Mar 31. Erratum in: PLoS Genet. 2011;7(5).
101. Wu W, Bhagat TD, Yang X, Song JH, Cheng Y, Agarwal R, Abraham JM, Ibrahim S, Bartenstein M, Hussain Z, Suzuki M, Yu Y, Chen W, Eng C, Greally J, Verma A, Meltzer SJ. Hypomethylation of noncoding DNA regions and overexpression of the long noncoding RNA, AFAP1-AS1, in Barrett's esophagus and esophageal adenocarcinoma. Gastroenterology. 2013;144(5):956–966.e4. doi:10.1053/j.gastro.2013.01.019.
102. Feber A, Xi L, Luketich JD, Pennathur A, Landreneau RJ, Wu M, Swanson SJ, Godfrey TE, Litle VR. MicroRNA expression profiles of esophageal cancer. J Thorac Cardiovasc Surg. 2008;135(2):255–60. doi:10.1016/j.jtcvs.2007.08.055. discussion 260.
103. Garman KS, Owzar K, Hauser ER, Westfall K, Anderson BR, Souza RF, Diehl AM, Provenzale D, Shaheen NJ. MicroRNA expression differentiates squamous epithelium from Barrett's esophagus and esophageal cancer. Dig Dis Sci. 2013;58(11):3178–88. doi:10.1007/s10620-013-2806-7.
104. Mathé EA, Nguyen GH, Bowman ED, Zhao Y, Budhu A, Schetter AJ, Braun R, Reimers M, Kumamoto K, Hughes D, Altorki NK, Casson AG, Liu CG, Wang XW, Yanaihara N, Hagiwara N, Dannenberg AJ, Miyashita M, Croce CM, Harris CC. MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res. 2009;15(19):6192–200. doi:10.1158/1078-0432.CCR-09-1467.

105. Leidner RS, Ravi L, Leahy P, Chen Y, Bednarchik B, Streppel M, Canto M, Wang JS, Maitra A, Willis J, Markowitz SD, Barnholtz-Sloan J, Adams MD, Chak A, Guda K. The microRNAs, MiR-31 and MiR-375, as candidate markers in Barrett's esophageal carcinogenesis. Genes Chromosomes Cancer. 2012;51(5):473–9. doi:10.1002/gcc.21934.
106. Fassan M, Volinia S, Palatini J, Pizzi M, Baffa R, De Bernard M, Battaglia G, Parente P, Croce CM, Zaninotto G, Ancona E, Rugge M. MicroRNA expression profiling in human Barrett's carcinogenesis. Int J Cancer. 2011;129(7):1661–70. doi:10.1002/ijc.25823. Epub 2011 Mar 11.
107. Revilla-Nuin B, Parrilla P, Lozano JJ, de Haro LF, Ortiz A, Martínez C, Munitiz V, de Angulo DR, Bermejo J, Molina J, Cayuela ML, Yélamos J. Predictive value of MicroRNAs in the progression of barrett esophagus to adenocarcinoma in a long-term follow-up study. Ann Surg. 2013;257(5):886–93. doi:10.1097/SLA.0b013e31826ddba6.
108. Hamano R, Miyata H, Yamasaki M, Kurokawa Y, Hara J, Moon JH, Nakajima K, Takiguchi S, Fujiwara Y, Mori M, Doki Y. Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the Akt signaling pathway. Clin Cancer Res. 2011;17(9):3029–38. doi:10.1158/1078-0432.CCR-10-2532.
109. Macha MA, Seshacharyulu P, Krishn SR, Pai P, Rachagani S, Jain M, et al. MicroRNAs (miRNA) as biomarker(s) for prognosis and diagnosis of gastrointestinal (GI) cancers. Curr Pharm Design. 2014;20(33):5287–97.
110. Sakai NS, Samia-Aly E, Barbera M, Fitzgerald RC. A review of the current understanding and clinical utility of miRNAs in esophageal cancer. Semin Cancer Biol. 2013;23(6 Pt B):512–21. doi:10.1016/j.semcancer.2013.08.005.
111. Varghese S, Lao-Sirieix P, Fitzgerald RC. Identification and clinical implementation of biomarkers for Barrett's esophagus. Gastroenterology. 2012;142(3):435–441.e2. doi:10.1053/j.gastro.2012.01.013.
112. Srivastava S. Cancer biomarker discovery and development in gastrointestinal cancers: early detection research network-a collaborative approach. Gastrointest Cancer Res. 2007;1(4 Suppl 2):S60–3.
113. Fouad YM, Mostafa I, Yehia R, El-Khayat H. Biomarkers of Barrett's esophagus. World J Gastrointest Pathophysiol. 2014;5(4):450–6. doi:10.4291/wjgp.v5.i4.450.
114. Davelaar AL, Calpe S, Lau L, Timmer MR, Visser M, Ten Kate FJ, Parikh KB, Meijer SL, Bergman JJ, Fockens P, Krishnadath KK. Aberrant TP53 detected by combining immunohistochemistry and DNA-FISH improves Barrett's esophagus progression prediction: a prospective follow-up study. Genes Chromosomes Cancer. 2015;54(2):82–90. doi:10.1002/gcc.22220.
115. Sato F, Jin Z, Schulmann K, Wang J, Greenwald BD, Ito T, Kan T, Hamilton JP, Yang J, Paun B, David S, Olaru A, Cheng Y, Mori Y, Abraham JM, Yfantis HG, Wu TT, Fredericksen MB, Wang KK, Canto M, Romero Y, Feng Z, Meltzer SJ. Three-tiered risk stratification model to predict progression in Barrett's esophagus using epigenetic and clinical features. PLoS One. 2008;3(4):e1890. doi:10.1371/journal.pone.0001890.
116. Jin Z, Cheng Y, Gu W, Zheng Y, Sato F, Mori Y, Olaru AV, Paun BC, Yang J, Kan T, Ito T, Hamilton JP, Selaru FM, Agarwal R, David S, Abraham JM, Wolfsen HC, Wallace MB, Shaheen NJ, Washington K, Wang J, Canto MI, Bhattacharyya A, Nelson MA, Wagner PD, Romero Y, Wang KK, Feng Z, Sampliner RE, Meltzer SJ. A multicenter, double-blinded validation study of methylation biomarkers for progression prediction in Barrett's esophagus. Cancer Res. 2009;69(10):4112–5. doi:10.1158/0008-5472.CAN-09-0028.
117. Alvi MA, Liu X, O'Donovan M, Newton R, Wernisch L, Shannon NB, Shariff K, di Pietro M, Bergman JJ, Ragunath K, Fitzgerald RC. DNA methylation as an adjunct to histopathology to detect prevalent, inconspicuous dysplasia and early-stage neoplasia in Barrett's esophagus. Clin Cancer Res. 2013;19(4):878–88. doi:10.1158/1078-0432.CCR-12-2880.
118. Hardikar S, Onstad L, Song X, Wilson AM, Montine TJ, et al. Inflammation and oxidative stress markers and esophageal adenocarcinoma incidence in a Barrett's esophagus cohort. Cancer Epidemiol Biomarkers Prev. 2014;23(11):2393–403.
119. Galipeau PC, Li X, Blount PL, Maley CC, Sanchez CA, Odze RD, Ayub K, Rabinovitch PS, Vaughan TL, Reid BJ. NSAIDs modulate CDKN2A, TP53, and DNA content risk for progression to esophageal adenocarcinoma. PLoS Med. 2007;4(2):e67.

120. Kostadinov RL, Kuhner MK, Li X, Sanchez CA, Galipeau PC, Paulson TG, Sather CL, Srivastava A, Odze RD, Blount PL, Vaughan TL, Reid BJ, Maley CC. NSAIDs modulate clonal evolution in Barrett's esophagus. PLoS Genet. 2013;9(6):e1003553. doi:10.1371/journal.pgen.1003553.
121. Bird-Lieberman EL, Dunn JM, Coleman HG, Lao-Sirieix P, Oukrif D, Moore CE, Varghese S, Johnston BT, Arthur K, McManus DT, Novelli MR, O'Donovan M, Cardwell CR, Lovat LB, Murray LJ, Fitzgerald RC. Population-based study reveals new risk-stratification biomarker panel for Barrett's esophagus. Gastroenterology. 2012;143(4):927–35.e3. doi:10.1053/j.gastro.2012.06.041.
122. di Pietro M, Boerwinkel DF, Shariff MK, Liu X, Telakis E, Lao-Sirieix P, Walker E, Couch G, Mills L, Nuckcheddy-Grant T, Slininger S, O'Donovan M, Visser M, Meijer SL, Kaye PV, Wernisch L, Ragunath K, Bergman JJ, Fitzgerald RC. The combination of autofluorescence endoscopy and molecular biomarkers is a novel diagnostic tool for dysplasia in Barrett's oesophagus. Gut. 2015;64(1):49–56. doi:10.1136/gutjnl-2013-305975.
123. Bennett M, Mashimo H. Molecular markers and imaging tools to identify malignant potential in Barrett's esophagus. World J Gastrointest Pathophysiol. 2014;5(4):438–49. doi:10.4291/wjgp.v5.i4.438.
124. Yates M, Cheong E, Luben R, Igali L, Fitzgerald R, Khaw KT, Hart A. Body mass index, smoking, and alcohol and risks of Barrett's esophagus and esophageal adenocarcinoma: a UK prospective cohort study. Dig Dis Sci. 2014;59(7):1552–9. doi:10.1007/s10620-013-3024-z.
125. Duggan C, Onstad L, Hardikar S, Blount PL, Reid BJ, Vaughan TL. Association between markers of obesity and progression from Barrett's esophagus to esophageal adenocarcinoma. Clin Gastroenterol Hepatol. 2013;11(8):934–43. doi:10.1016/j.cgh.2013.02.017.
126. Keszei AP, Schouten LJ, Driessen AL, Huysentruyt CJ, Keulemans YC, Goldbohm RA, van den Brandt PA. Vegetable, fruit and nitrate intake in relation to the risk of Barrett's oesophagus in a large Dutch cohort. Br J Nutr. 2014;111(8):1452–62. doi:10.1017/S0007114513003929.
127. Lao-Sirieix P, Boussioutas A, Kadri SR, O'Donovan M, Debiram I, Das M, Harihar L, Fitzgerald RC. Non-endoscopic screening biomarkers for Barrett's oesophagus: from microarray analysis to the clinic. Gut. 2009;58(11):1451–9. doi:10.1136/gut.2009.180281.
128. Ross-Innes CS, Debiram-Beecham I, O'Donovan M, Walker E, Varghese S, Lao-Sirieix P, Lovat L, Griffin M, Ragunath K, Haidry R, Sami SS, Kaye P, Novelli M, Disep B, Ostler R, Aigret B, North BV, Bhandari P, Haycock A, Morris D, Attwood S, Dhar A, Rees C, Rutter MD, Sasieni PD, Fitzgerald RC, BEST2 Study Group. Evaluation of a minimally invasive cell sampling device coupled with assessment of trefoil factor 3 expression for diagnosing Barrett's esophagus: a multi-center case-control study. PLoS Med. 2015;12(1):e1001780. doi:10.1371/journal.pmed.1001780.
129. Bansal A, Lee IH, Hong X, Mathur SC, Tawfik O, Rastogi A, Buttar N, Visvanathan M, Sharma P, Christenson LK. Discovery and validation of Barrett's esophagus microRNA transcriptome by next generation sequencing. PLoS One. 2013;8(1):e54240. doi:10.1371/journal.pone.0054240.
130. Vega KJ, May R, Sureban SM, Lightfoot SA, Qu D, Reed A, Weygant N, Ramanujam R, Souza R, Madhoun M, Whorton J, Anant S, Meltzer SJ, Houchen CW. Identification of the putative intestinal stem cell marker doublecortin and CaM kinase-like-1 in Barrett's esophagus and esophageal adenocarcinoma. J Gastroenterol Hepatol. 2012;27(4):773–80. doi:10.1111/j.1440-1746.2011.06928.x.
131. Whorton J, Sureban SM, May R, Qu D, Lightfoot SA, Madhoun M, Johnson M, Tierney WM, Maple JT, Vega KJ, Houchen CW. DCLK1 is detectable in plasma of patients with Barrett's esophagus and esophageal adenocarcinoma. Dig Dis Sci. 2015;60(2):509–13. doi:10.1007/s10620-014-3347-4.
132. Ahmad J, Arthur K, Maxwell P, Kennedy A, Johnston BT, Murray L, McManus DT. A cross sectional study of p504s, CD133, and Twist expression in the esophageal metaplasia dysplasia adenocarcinoma sequence. Dis Esophagus. 2015;28(3):276–82. doi:10.1111/dote.12181.
133. Howlader N, Noone AM, Krapcho M, et al., editors. SEER cancer statistics review. Bethesda, MD: National Cancer Institute; 1975–2010.

Chapter 13
Common Variants Confer Susceptibility to Barrett's Esophagus: Insights from the First Genome-Wide Association Studies

Claire Palles, John M. Findlay, and Ian Tomlinson

Introduction

Barrett's esophagus (BO), also known as columnar-lined esophagus is one of the most common esophageal disease processes and, due to its strong malignant potential, one of the most important. It involves the replacement of a variable area of the normal esophageal squamous epithelium by endoscopically visible columnar epithelium [1, 2].

The prevalence in Western countries is estimated to be 1–5 % in unselected patients undergoing endoscopy [3, 4] or *postmortem* examination [5]. A number of clinical risk factors have been identified including male gender, Caucasian ethnicity, a positive family history, increasing age, obesity, and smoking. BO is associated with a dramatically increased risk of developing esophageal adenocarcinoma (OAC). Estimates of the heightened risk of OAC in BO patients compared to the general population are broad; ranging from an 11 to 125 % increase [6, 7]. Risk increases progressively with length of the Barrett's segment, and the presence and grade of dysplasia. BO is not associated with squamous cell carcinoma of the esophagus (OSCC), which is historically and globally the commonest histological

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subtype. However, in countries such as the UK, USA, and Australia the incidence of OAC has changed profoundly in just a few decades, from what was once a rare condition to by far the most common subtype of esophageal cancer [8]. Compared to other solid tumors the unselected 5-year survival rate of OAC is very poor (<20 % compared to 51 % for colorectal cancers; data from the national cancer intelligence network (NCIN)). Prognosis may be little better for patients treated with maximal oncological and surgical therapy due to early and aggressive metastatic behavior and is therefore profoundly influenced by stage of detection [9, 10]. Early OAC is asymptomatic, and while it may be detected fortuitously in patients with known BO undergoing surveillance, many patients with BO are also asymptomatic and therefore undiagnosed [11]. Consequently, in the absence of a feasible population strategy for either condition, most patients with OAC present late to be faced with limited and poorly affected treatment options. There is therefore a real need to identify patients at risk of developing BO.

Although the majority of BO cases appear to be sporadic [12], a small number of studies report familial clustering of cases suggesting an underlying genetic predisposition. A total of 7–10 % of BO cases demonstrate such clustering, and have been identified as familial BO, defined as cases with at least one first- or second-degree relative with BO or OAC [13, 14]. The risk of BO and OAC in relatives of affected cases has been estimated to be increased by two- to fourfold [15, 16]. However "familial" and "sporadic" BO are clinically indistinguishable, so whether these actually represent distinct phenotypes is not clear.

BO is strongly associated with chronic gastroesophageal reflux disease (GORD), a complex multifactorial condition encompassing both endoscopy-negative symptomatic reflux and esophagitis (which may be asymptomatic). Excessive exposure of esophageal squamous epithelium to gastroduodenal reflux is the primary driver of columnar metaplasia; however, only a minority of patients with such exposure will develop BO, indicative of the complexity inherent in this process. A study of GORD in 1960 monozygotic and dizygotic twins estimated that 43 % (95 % confidence interval (CI) 32–55 %) of the variation in risk of GORD was due to genetic factors [17]. No similar twin studies of BO have been performed but given the association between GORD and BO this is suggestive that a substantial proportion of the susceptibility to BO could also be explained by genetic factors. A segregation analysis of 881 pedigrees of "familial" BO suggested that risk could be best explained by a model of dominant inheritance with a polygenic component [18].

Evidence from the epidemiology studies described earlier motivated many candidate gene and genetic linkage studies of BO. Genetic linkage studies have identified highly penetrant genes predisposing to Mendelian cancer syndromes such as adenomatous polyposis coli colorectal cancer syndrome (*APC* [19]) and Hereditary Nonpolyposis Colorecal Cancer (HNPCC) (*MSH2* and *MLH1* [20, 21]), but no such genes have been identified for BO or OAC [22]. The failure of linkage studies of BO supports the polygenic model of disease risk where multiple low penetrance variants, each having small effects, combine additively or mutiplicatively to confer individual risk. Alleles with modest effects (altering disease risk by less than twofold) are very difficult to detect using linkage approaches.

Many candidate alleles of varying frequencies have been investigated in relation to BO risk [22–37]. No reproducible, convincing associations have been identified in candidate gene studies, apart from perhaps rs909253 which maps to a highly conserved base in an intron of *LTA* (*lymphotoxin-alpha*, also known as *tumor necrosis factor-β*). This SNP was first tested for an association with BO risk by Menke et al. [33] in 257 cases and 197 healthy controls. In silico replication in the first genome-wide association study of BO [38] showed evidence of a nominally significant association at $P=0.005$. Additional evidence of replication was seen in the second phase of this study where cases and controls were genotyped using the Immunochip ($P_{meta}=3.1\times10^{-4}$, odds ratio (OR) = 1.07). The sample sizes used to investigate candidate genetic variants in relation to BO risk have, however, been small. To date no candidate gene study of BO susceptibility has exceeded 250 cases and 250 controls and hence they have been insufficiently powered to detect even common variants with a minor allele frequency (MAF) of 30 %, if their true OR is less than 1.5.

In 2012, the first genome-wide association study (GWAS) of BO was performed in 1852 cases and 5172 controls [39]. This agnostic approach identified two loci associated with BO. A subsequent follow-up study increased the number of identified BO susceptibility loci to 4 [38]. Four other loci were identified in a separate GWAS, combining both BO and OAC cases [38, 40]. The genes suggested as the targets at these loci are discussed in detail in this chapter and they implicate regulation of the immune system and transcription factors involved in esophageal development in susceptibility to BO/OAC and the limited overlap of susceptibility loci for BO/OAC and OSCC confirms that ESC and OAC are two distinct diseases with different genetic origins.

Before looking at the results of both GWASs, we briefly review the reasons for conducting a GWAS and the requirements for successfully conducting one. We also look at how progress in understanding of the genetic variants that contribute to BO/OAC susceptibility compares to other cancers.

Genome-Wide Association Studies: Motivation and Practicalities

Two hypotheses have motivated genome-wide association studies of common complex diseases: the common disease/common variant hypothesis (CD/CV) [41, 42] and the belief that common diseases are polygenic. The basis of the CD/CV hypothesis is that a small number of common variants can explain an individual's risk of common complex diseases. The polygenic model of disease makes no assumption as to the frequency of the disease-associated variants, but proposes that a large number of cases occur in a small susceptible proportion of the population who carry multiple such variants [43].

The result of the International Hap Map Project [44] was a comprehensive catalog of the sites in the human genome that vary in populations with African, Asian, or European ancestry. The most recent release, phase 3, of the project contains genotypes for almost two million single nucleotide polymorphisms (SNPs). These have been used to generate high-resolution genome-wide linkage disequilibrium (LD) maps from which sets of SNPs that capture variation within LD blocks can be selected (tagging SNPs). With the development of high throughput, high accuracy, low cost SNP genotyping methods such as the BeadArray platform from Illumina [45] it became feasible to genotype hundreds of thousands of tagging SNPs in large numbers of samples. Early GWAS studies made use of panels with up to 500,000 SNPs each with a MAF greater than 10 %. Thus, it became possible to quantify what proportion of an individual's risk of common diseases could be explained by common genetic variants.

Since 2005 and the publication of the first GWAS (of age-related macular degeneration) over 8000 common genetic variants have been associated with 750 different traits (data from the NHGRI Catalogue of Genome Wide Association studies, updated in August 2014). Figure 13.1 demonstrates the profound impact that the GWAS approach has had upon our understanding of the common variation in the human genome that determines traits such as hair color, height, and blood pressure as well risk of common diseases such as diabetes and cancer. Each colored circle on Fig. 13.1 represents a locus associated with the trait of interest at $P < 5 \times 10^{-8}$ (the conventional significance threshold required when conducting studies testing many hundreds of thousands to millions of markers).

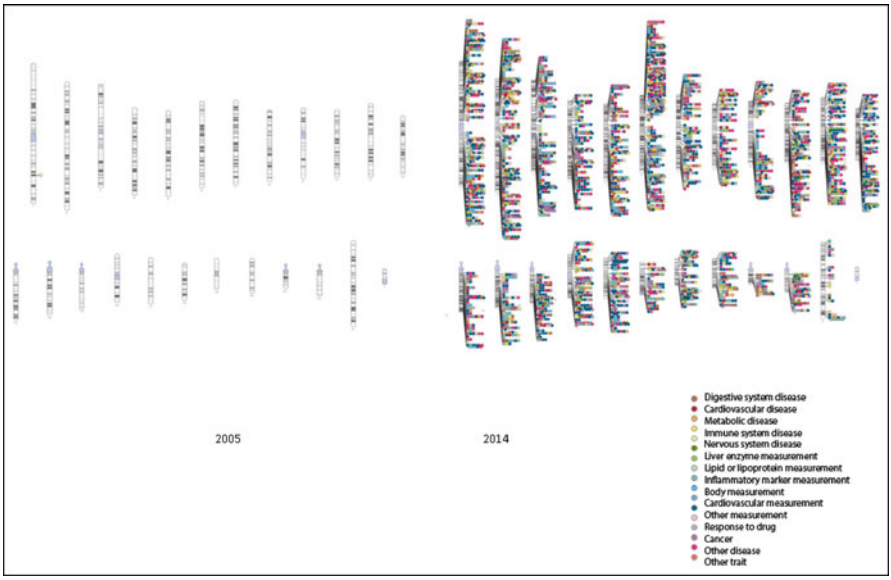

Fig. 13.1 The impact of Genome-wide association studies since the first published study in 2005. Data from the NHGRI GWAS catalog (www.genome.gov/gwasstudies)

A single GWAS typically consists of over 1000 cases and 1000 controls; however, far larger sample sizes have been necessary to detect many of the variants in the NHGRI catalog. This is because the effect sizes of individual variants identified using this approach have been small. Only three out of the 299 common variants (MAF>5 %) in the NHGRI catalog associated with cancer risk have an OR >2. Rather, the mean OR of these 299 variants is much smaller at 1.22 (range 1.05–2.63). Indeed, it was only possible to identify the smallest effect size variants in the catalog, by meta-analysis of 9 independent GWAS studies with a combined sample size of 10,052 breast cancer cases and 12,575 controls of European ancestry [46]. Most GWAS employ a multistage approach to maximize the numbers of samples that can be genotyped; a subset of samples are analyzed on a genome-wide genotyping platform (discovery phase), SNPs with the best evidence of association are taken forward for focused genotyping in the remainder of samples (replication phases).

GWAS is now considered a standard genetic approach in the study of human diseases, but ensuring a high data quality remains a considerable undertaking. This is evidenced by the best practice guidelines available to guide the way these studies are designed and conducted [47–50]. The process of genotyping hundreds of thousands of markers in many thousands of cases and controls permits and demands quality control procedures to ensure:

1. Correct sample handling and identity.
2. Exclusion of related individuals (relatives can be identified by identity by descent).
3. Removal of poorly genotyped samples or SNPs (with call rates <95 %).
4. Removal of samples that have a different ancestry to the population of study.

For criterion 4, principal component analysis (PCA) of genetic markers that are not in LD with one another is used to examine the variation between samples in a study, to determine if cases and controls originated from the same population [51]. Testing—and if necessary controlling—for population stratification or population structure (i.e., differences in allele frequencies in subpopulations) helps to avoid reporting spurious disease associations caused by ancestry differences between cases and controls. To this end, global test statistics can be analyzed to check for genomic inflation (λ values different from 1). As discussed, identification of many of the GWAS signals in the NIHR catalog was only possible with very large numbers of samples. In many studies cases and controls were ascertained from multiple countries, particularly for rare conditions. Principal component analysis has been essential in controlling for population structure in such studies.

Early GWAS studies made use of panels with up to 500,000 SNPs, but current genotyping arrays on the market include up to five million markers, many of which were discovered by the 1000 Genomes Project [52] which has sequenced over >1000 individual genomes at low depth (4×), with the latest release of data (June 2014) containing 36,820,992 SNPs. New generation SNP genotyping arrays therefore contain both tagging SNPs and rare variants that were poorly tagged by initial genotyping arrays used at the start of the GWAS era. Recently, studies have been published that have made use of exome content arrays to systematically assess the

contribution of coding variants to disease risk [53, 54]. However, again unless the protein and phenotypic effects of coding variants are major, large sample sizes are still required, thus genotyping on arrays is (at present) far more economical than exome sequencing. Indeed, the two exome array papers referenced earlier genotyped 40,000 and 150,000 individuals, respectively, with the variants identified being of "mini-effect."

Genotype data from both the 1000 Genomes and International HapMap Projects have been phased into haplotypes for use as reference sets by researchers with tagging SNP data on samples of their own [55]. Imputation methods allow SNPs that have not been genotyped in these samples, but are present in the reference sets to be inferred with a high degree of accuracy, thereby greatly expanding the number of variants that can be examined in association studies. Imputation has been particularly useful in studies involving consortia and in meta-analyses where individual datasets genotyped on different arrays have been imputed to generate a common and combined set of SNPs [46]. Imputation strategies using additional local reference panels (generated either by genotyping a subset of samples on a high density array or converting next-generation sequence data into phased genotypes) in addition to the 1000 Genomes data have shown greater accuracy, particularly for lower frequency SNPs (MAF <5 %) [56, 57]. The ability to impute additional variants in loci associated with disease helps to refine associations and for a minority of disease loci has allowed the underlying functional variant to be identified [58].

Genome-Wide Association Studies Have Identified 8 Loci Associated with Risk of Barrett's esophagus

Two GWAS of BO have been performed to date. The first, published in 2012, was part of the Wellcome Trust Case Control Consortium 2 (WTCCC2) study of 15 common diseases [39]. A total of 5172 UK population-based controls from the 1958 Birth cohort (58C) and National Blood Service (UKBS) were analyzed alongside 1852 cases from the Aspirin and Esomeprazole Chemoprevention Trial of Cancer in Barrett's esophagus (AspECT). Replication samples were obtained from the CHemoprevention Of Premalignant Intestinal Neoplasia (ChOPIN) study, Esophageal Adenocarcinoma GenEtics Consortium (EAGLE) and Barrett's and Esophageal Adenocarcinoma Consortium (BEACON). A total of 5986 cases and 12,825 were used in the replication phase. A follow-up study where additional SNPs were prioritized for replication was published in 2015 [38]. This study included an additional 1423 cases and 2028 controls not genotyped in the original publication.

In 2013, a GWAS combining OAC and BO was published by Levine et al. on behalf of BEACON [40]. The discovery phase consisted of 1516 OAC cases, 2416 BO cases, and 3209 controls. The cases and 2187 of the controls were from case–control studies conducted in Western Europe, Australia, and North America by investigators in BEACON. The additional 1022 cancer-free controls were obtained

from a study of melanoma [59]. The 8 loci (9 SNPs) that reached genome-wide significance in the BO-only GWAS, BO, and OAC GWAS or the post-GWAS follow-up study are shown in Table **13.1** and are discussed in detail as follows.

Meta-Analysis of BO/OAC GWASs Has Been Limited to the Top Association Signals in Either Study

Discovery phase BEACON BO data were included in a subsequent meta-analysis of the top hits reported in Su et al. [39] and Palles et al. [38]. Data on the 3 genome-wide significant loci and 87 other SNPs with $P_{assoc} < 10^{-4}$ from the Levine GWAS [40] were meta-analyzed with discovery phase data from the WTCCC2 GWAS (although, as some controls were shared between stages in both GWAS, a separate control population was drawn from existing colorectal cancer data). Global genome-wide meta-analysis of the two GWAS studies has yet to be performed.

In the meta-analysis of BO only cases, one of the three significant loci reported by Levine et al. to be associated with BO/OAC (but not BO alone) reached genome-wide significance: rs2687201, near *FOXP1*. In the combined meta-analysis of all BO and OAC cases, all 4 SNPs identified by Levine et al. as genome-wide significant remain so, but the WTCCC2 discovery data only provided support for 3 ($P < 0.07$). Indeed, there was no evidence of association between one of the *CRTC1* SNPs and BO, rs10419226, fixed-effects OR: 1.01 (95%CI 0.91–1.11), $P = 0.87$. However, the fixed effects meta-analysis of this SNP showed evidence of heterogeneity and so a random effects meta-analysis was performed; the result of this was a nonsignificant association with BO/OAC. The reasons for the heterogeneity are unclear and so it is ultimately difficult to draw a conclusion on this SNP. By contrast, the strength of evidence for the other SNP in *CRTC1* (rs10423674) and the SNPs in *BARX1* and *FOXP1* (rs11789015 and rs2687201) was improved upon meta-analysis. In addition through this process, a new SNP was identified (from the top 87 SNPs reported by Levine et al.) as being associated with BO and OAC at genome-wide significance, mapping to *ALDH1A2* (Table **13.1**).

How Progress in Identification of Loci Associated with Barrett's Esophagus and Esophageal Adenocarcinoma Compares with Progress for Other Selected Cancer Types

Table 13.2 shows the number of loci that have been identified by GWAS studies of breast, colorectal, OSCC, OAC, and BO. As can be seen the numbers of BO and OAC samples analyzed to date are much lower than the numbers included in studies of breast and colorectal cancers, where 90 and 32 independent loci have been identified and replicated, respectively, in European populations. In particular, the

Table 13.1 Genome-wide significant germline variants associated with Barrett's Esophagus or Barrett's Esophagus and esophageal adenocarcinoma

Variant	Gene	Association results			Cases	Reference
		Effect allele	MAF (cases/controls)	OR (95 % CI), *P*		
rs9257809 rs9936833	MHC locus *FOXF1*	A C	0.1/0.13 0.42/0.38	1.21 (1.13–1.28), 4.09×10^{-9} 1.14 (1.10–1.19), 2.74×10^{-10}	BO only BO only	[39] [39]
rs10419226 rs10423674 rs11789015 rs2687201	*CRTC1* *CRTC1* *BARX1* *FOXP1*	A T G T	0.49/0.46 0.30/0.34 0.26/0.28 0.34/0.30	1.14 (1.09–1.19), 0.04[a] 0.88 (0.84–0.91), 4.87×10^{-11} 0.85 (0.81–0.89), 1.14×10^{10} 1.17 (1.11–1.23), 6.70×10^{-10} 1.16 (1.10–1.23), 4.61×10^{-8}	BO and OAC BO and OAC BO and OAC BO and OAC BO only	[38, 40] [38, 40] [38, 40] [38, 40]
rs3072 rs2771108 rs3784262	*GDF7* *TBX5* *ALDH1A2*	G G G	0.41/0.36 0.38/0.41	1.14(1.09–1.18), 1.75×10^{-11} 0.90(0.86–0.93), 7.48×10^{-9} 0.90(0.87–0.93), 3.72×10^{-9}	BO only BO only BO and OAC	[38, 40] [38, 40]

[a]Random effects meta-analysis was performed for this SNP because of the significant heterogeneity observed in the fixed effects analysis

genome-wide discovery phases are markedly smaller. The combined genome-wide sample sizes of the two GWAS of BO and BO/OAC above are 3368 cases and 8381 controls, compared to 15,748 breast cancer cases and 18,084 controls. This has inevitably restricted discovery of BO-associated variants to those with larger effect sizes (OR >1.1) [47].

Lack of Evidence for the Involvement of Esophageal Squamous Cell Carcinoma Loci in Barrett's Esophagus and Esophageal Adenocarcinoma, Apart from Those at the Major Histocompatibility Complex

Considerably more loci have been reported in GWAS of OSCC and a logical question is whether any of these loci may also predispose to BO or OAC. As shown in Table 13.2 there are 18 loci reported to be associated with OSCC; 14 in main effects analysis of GWAS studies and 4 in high-risk subsets. However, replication of OSCC GWAS hits has been challenging, with many SNPs showing evidence of heterogeneity. This has

Table 13.2 How progress in identification of loci associated with Barrett's esophagus and esophageal adenocarcinoma compares with progress for other selected cancer types

Disease	GWAS sample size cases:controls	Replication sample size cases:controls	Total number of risk loci identified to date	% of variation explained by identified variants	Population	Reference
Breast cancer	15,748:18,084	46,785:42,892	90	~16%	European Caucasian	[103]
Colorectal cancer	5626:7817	14,037: 15,937	32[a]	~7%	European Caucasian	[104]
	2627:3797 (JPN) 1894:4703 (AFR)	16,823:18,211 (EUR)			Japanese and African GWAS European replication	[105]
	2098: 6172 (CHN)	3519:6275 (CHN) 2814:11,358 (JPN) 6532:8140 (CHN, JPN, SKO)			Asians from China, Japan, and South Korea	[106]
Esophageal squamous cell carcinoma	5337:5787 (CHN) 182:927 (JPN)	9654:10,058 (CHN) 782: 1898 (JPN)	18[b]	Not stated	Asians from China and Japan	[107] [108]
Esophageal adenocarcinoma	1516:3209[c]	874:6911[d]	4		European Caucasians	[40]
Barrett's esophagus	1852:5172	5986:12,825	4		European Caucasians	[39]

[a]Number of independent loci replicable in European Caucasians

[b]14 Loci significant in main effects analyses

[c]Cases were analyzed in conjunction with 2416 Barrett's esophagus cases. Four loci were significant in the BO and OAC analyses

[d]Cases were analyzed with 759 Barrett's esophagus cases

mainly been attributed to different environmental exposures in the populations tested. Four out of the 14 reported loci showed no signal in a joint analysis of three Chinese GWAS of over 5000 cases and controls from Beijing, Shanxi, or Henan (17q21.3, 17p13.3, 20p13, and 18p11.21, $P<0.1$). The replicated SNPs in loci where *PLCE1*, *PDEAD*, *CASP8*, *ST6GAL1,* and *C20orf54* have been suggested as the targets were directly genotyped on the discovery arrays used in the BO GWAS described by Su et al. [39], but none showed sufficient evidence of association to be prioritized for replication. Imputation of the discovery BO cases and controls showed no evidence of association at the other OSCC loci where *CHEK2, RUNX1,* and *HEATR3* have been suggested as the targets.

The missense variant in *PLCE1*, rs2274223, implicated in susceptibility to OSCC has also been identified in a GWAS of gastric cardia adenocarcinoma (GCA) in an ethnic Chinese population [60]. As OSCC and GCA in this population share environmental risk factors and geographical distribution it might be expected that they also share genetic risk factors. However, this is in stark contrast to GCA in Western populations, in whom GCA and OAC are much more biologically and clinically related. In these populations, smoking is the only clear environmental risk factor shared by OSCC and BO and OAC, which are markedly different as regards geographical distribution, histology, and pathophysiology. This reinforces the need to consider discrete populations, both at the level of ethnic populations and also geography, and ultimately limits meaningful comparison between these essentially discrete disease processes.

This notwithstanding, one potentially overlapping and intriguing GWAS signal in studies of OSCC and BO/OAC has been identified, mapping to Chr6p21. SNPs at chr6p21.1 and chr6p21.3 have been identified as susceptibility loci for BO and OSCC, respectively, with the former (rs9257809 at 6p21.1) subsequently found to also be associated with OAC in a Dutch study of 431 patients with OAC and 605 healthy controls [61]. Chr6p21 is within the Major histocompatibility complex where long-range LD makes it difficult to identify the underlying genes. There are 121 functional genes within the MHC; most of these have roles in antigen presentation to T-cells or encode cytokines. MHC genes are associated with the response to many infectious diseases and their dysregulation is known to contribute to common auto-immune diseases such as Systemic Lupus Erythematosus and Crohn's disease, in addition to contributing to the survival of cancer cells. Loss of Class I MHC molecules prevents peptide presentation to cancer-specific T-cells and natural killer (NK) cells, enabling immune system evasion. HLA gene variants have been reported to be associated with lung, nasopharyngeal, and cervical cancers [62–64]. *HLA-DRB1* and *HLA-DQA1* flank the OSCC GWAS SNP and *OR2D12* and *OR2D13* flank the BO GWAS SNP (Fig. 13.2) but each SNP is in strong LD with SNPs across a 1–2 Mb and the target gene at either locus remains unknown. The OSCC and BO associated SNPs are 3.2 Mb apart and were detected in studies of differing ethnicity and so are unlikely to tag the same functional SNP, but these findings do however suggest a role for the immune response in both BO and OSCC.

A variable inflammatory response to gastroduodenal reflux is a major driver of reflux esophagitis and a strong risk factor for BO. The traditional model of epithelial

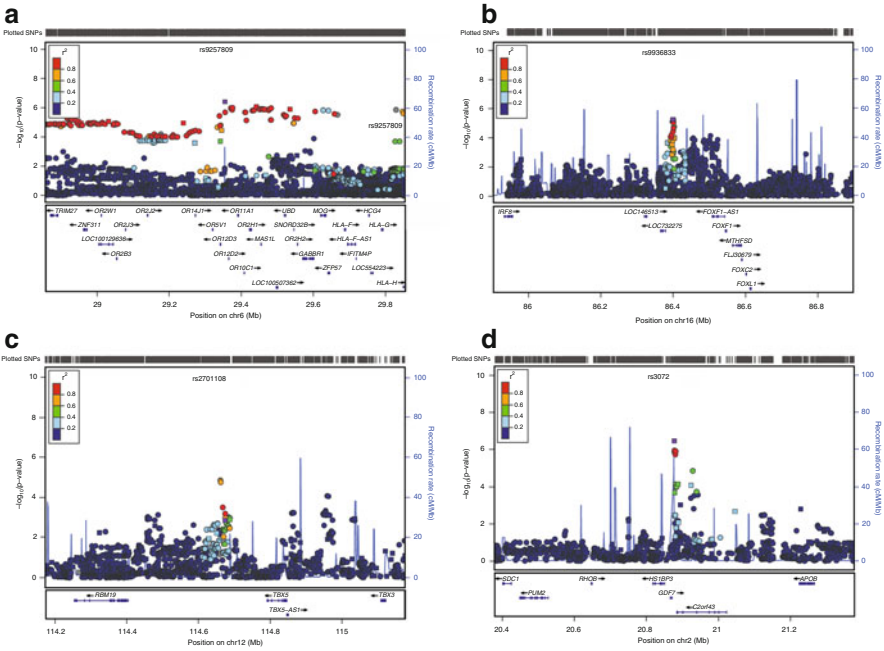

Fig. 13.2 Regional association plots for SNPs associated with Barrett's esophagus in the discovery phase of the GWAS described by Su et al. [39]. Regional association plots were generated using LocusZoom (http://csg.sph.umich.edu/locuszoom/). The r^2 values are from the 1000 Genomes Project CEU 2010 data. Imputed SNPs are indicated by squares and genotyped SNPs are indicated by circles. Recombination data (shown in *light blue*) is from the CEU individuals of the HapMap project. (**a**) Chr6p21, (**b**) Chr16q22, (**c**) chr12q24, (**d**) chr2p24

damage is that gastric acid and pepsin from the stomach, in synergy with bile acids from the duodenum, directly injure the squamous epithelium of the esophagus, resulting in cellular necrosis, apoptosis, and often frank esophagitis. However, there is often poor correlation between the amount and duration of reflux and clinical symptoms of epithelial damage, which suggests a greatly more complex pathophysiology. A number of human and animal studies have identified a large number of contributory factors and antireflux mechanisms, all of which may be modulated by genomic variants. These include mechanical mechanisms (such as muscle tone at the lower esophageal and diaphragmatic sphincters), esophageal acid clearance (such as salivary buffering and peristalsis), and intrinsic epithelial defenses (such as a transmural electrochemical gradient, tight cell junctions, pH-dependent cation channels, and intracellular buffers), in conjunction with dietary and pharmacological factors and raised intra-abdominal pressure due, for example, to obesity [65]. However, a variable inflammatory response to reflux, involving cytokines released from the squamous cells and inflammatory infiltrate has been implicated in potentiating cellular damage [66]. In addition, such inflammation can inadvertently increase reflux and epithelial damage, via impairment of esophageal motility and

sphincter function; cytokines such as interleukin (IL) 1B, IL6, and IL8 impair the former in vitro [67, 68], with IL1-B and IL-6 inducing prostaglandin E2 (PGE2)-mediated lower esophageal sphincter relaxation [69, 70].

Reflux-associated damage is normally repaired by squamous epithelial replacement, but in some individuals it is rather replaced by metaplastic, specialized intestinal-like columnar epithelium: BO. While reflux is a prerequisite for BO, chronic intermittent exposure of the distal esophagus to acid is universal [71], but only a few patients with GORD develop BO, highlighting a complex and only partially understood process. It is thought that molecular differences between individual responses to reflux-mediated damage may partially determine whether metaplasia occurs, and indeed a similarly variable progression from metaplasia, via dysplasia to cancer [72, 73]. Genes in the MHC are very plausible candidates that may determine these differential responses between individuals. Work to uncover the genes responsible for the associations at Chr6p21 will further our understanding of the genesis of esophageal metaplasia and carcinoma.

One of the newly identified loci in OSCC adds further evidence for a comparable involvement of the immune response in OSCC. The associated variant is a synonymous SNP that maps to the coding region of transmembrane protein 173 (*TMEM173*), which promotes the production of type 1 interferon. A SNP in LD with the associated SNP has been identified as an expression quantitative trait locus (eQTL) in lymphoblastoid cells where it is associated with allele specific expression of genes in segment AC135457.2 [74]. *SLC23A1* (encoding the sodium-dependent vitamin C transporter) is within this region, with low vitamin C levels previously implicated in risk of OSCC [75].

As discussed earlier, results of GWASs of OSCC and OAC to date suggest that the genetic risk factors for these two forms of esophageal cancer are different, with the exception of genetic determinants of the immune response.

The Involvement of Alcohol Dehydrogenase Genes in OSCC and BO/OAC

Two of the OSCC loci that show strong gene–environment interactions and have only been identified and replicated in populations with strong alcohol and smoking-related risks [76] map to *ALDH2* and *ADH1B*. The A allele of rs671 in *ALDH2* (importantly not observed in Northern and Western European (CEU) populations, where reference allele G is invariant) leads to production of a catalytically inactive form of the enzyme. Homozygous carriers are unable to oxidize acetaldehyde and heterozygotes have low activity [77]. The variant allele of rs1229984 was reported to exhibit 30- to 40-fold lower enzymatic activity for ethanol oxidation than *ADH1B*1* [78]. Alcohol is a risk factor for OSCC independent of these two genotypes, but in individuals with one or both variant alleles acetaldehyde exposure is

greater as the rate of clearance is now much lower. The mechanism whereby alcohol increases carcinogenesis has not been fully uncovered, but carcinogenic acetaldehyde is thought to cause DNA damage and the number of acetaldehyde DNA adducts was found to be sevenfold higher in heavy drinkers compared to nondrinkers [79]. Chronic alcohol intake has been shown to reduce levels of the signaling molecule retinoic acid (RA), which is involved in cellular differentiation, proliferation, and apoptosis [80]. Enzymes such as CYP26A1 (cytochrome P450, family 26, subfamily A, polypeptide 1) which are responsible for breaking down retinoic acid (RA) have been shown to have an oncogenic effect [81].

Interestingly, a SNP in *ALDH1A2* (which is also a member of the alcohol dehydrogenase family) has recently been identified as a susceptibility allele in a joint analysis of BO and OAC. *ALDH1A2* encodes retinaldehyde dehydrogenase 2, which catalyses the synthesis of retinoic acid (RA), a metabolite of vitamin A, and may also be involved in alcohol metabolism. While heavy drinking is a known risk factor for OSCC, the effect of alcohol consumption on the risk of BO and OAC is less clear and inverse associations between alcohol consumption and risk of BO and OAC have been reported [82]. Perhaps the association between *ALDH1A2* genotype and BO/OAC risk might be due to its effect upon RA synthesis rather than its role in alcohol metabolism. The genes associated with OSCC are known to encode the enzymes mainly responsible for the conversion of alcohol to harmless acetate, although the role of ALDH1A2 in alcohol metabolism is less well known.

In embryonic development RA acts as a ligand that binds to retinoic acid receptors (RARs) which in turn act as transcription factors binding to retinoic acid responsive elements (RAREs) in the DNA of target genes, activating transcription. RA expression is tightly regulated in embryonic tissues by the spatial expression patterns of the enzymes that synthesize and catalyze it [83]. Confusingly, RA is involved in squamous esophageal epithelial embryogenesis during foregut development, yet is overexpressed in BO. Incubation of either squamous epithelium (SE) biopsies or squamous esophageal cell lines with RA leads to gene expression and morphology changes reminiscent of BO [84]. It has been suggested that these contrasting effects of RA might be explained by differential expression of subtypes of RARs. RAR-alpha and RXR-gamma have been shown to be up-regulated and RAR-gamma and RXR-beta down-regulated in BO compared to SE. While RA is overexpressed in BO, expression is reduced during the process of malignant transformation to BO dysplasia and OAC, consistent with its role in tumor suppression. It remains to be determined whether the SNP in *ALDH1A2* which shows an association with BO and OAC susceptibility, or SNPs in LD with it, can alter expression of *ALDH1A2* and whether this affects levels of RA. Given the overexpression of RA in nondysplastic BO it would be interesting to examine this SNP in a cohort of BO cases that never progressed to dysplasia and EAC. It would also be interesting to know whether there is any association of this SNP with OSCC. The minor allele of this variant is G in Caucasian populations, but A in Asians. The G allele was found to be protective in the GWAS of BO and OAC.

The Other Non-MHC Loci Associated with Risk of BO Map to Potential Enhancer Regions That May Regulate Transcription Factors Involved in Esophageal Development

Three non-MHC loci have been identified at genome-wide significance in BO cases, chr16q24, chr2p24, and chr12q24 [38, 39]. rs9936833 at chr16q24 was also shown to be associated with risk of OAC [61]; the SNPs at the other loci have not yet been tested in OAC cases. The tagging SNPs in each locus map to, or are in LD with, SNPs in potential intergenic enhancer regions adjacent to genes encoding transcription factors with roles in esophageal development. None of them are in strong LD ($r^2 > 0.4$) with nonsynonymous SNPs in the nearby genes, suggesting that the functional variants in these loci may affect gene expression and regulation rather than protein sequence. None of the SNPs are eQTLs in publically available data, but it should be noted that the relevant tissue type for comparison (i.e., embryonic esophageal tissue) has not been tested.

FOXF1

The first locus to be identified was marked by rs9936833 and as shown in Table **13.1** the C allele of this variant is associated with an increased risk of BO. This intergenic SNP maps 24 kb 5′ to a long nonprotein coding RNA LINC00917 (previously known as LOC732275) and 141 kb 5′ of *FOXF1*, a member of the forkhead family of transcription factors. FOXF1 is downstream of the Hedgehog (HH) signaling pathway, which is an essential determinant of foregut separation. Disruption of FOXF1 causes a similar phenotype as a reduction or abolishment of HH signaling. Mice heterozygous for a *foxf1* null allele had major structural abnormalities, including a narrow esophageal lumen, aberrant connection to the trachea (tracheoesophageal fistula) and failure of the esophagus to join to the stomach (esophageal atresia) [85]. As can be seen in Fig. 13.2b, imputation of ungenotyped SNPs in a 1 Mb region centered on rs9936833 did not identify any other SNPs with evidence of a stronger association. There are however multiple SNPs in strong LD with rs9936833 that are all associated with BO risk (these SNPs span a region of ~40 kb). One such SNP, rs1979654 ($r^2 = 0.79$, $D' = 0.98$), is the most plausible functional variant in the locus based upon examination of publically available data. rs1979654 lies in a DNase 1 hypersensitive cluster identified by ENCODE in multiple cell lines (UCSC Genome Browser data). The region rs1979654 that maps to is also marked by histones associated with enhancer regions (H3K4Me1 and H3K27Ac) and is predicted to affect binding of multiple transcription factors including STAT3, MYC and FOXA1. There is also a canonical T box binding site and a Gli binding site in the 40 kb region marked by rs9936833. The sites are within one kb of each other and *tbx5* and *gli1* have been shown to regulate *foxf1* in a mouse model [86]. It would be interesting to see whether the region containing rs9936833 or rs1979654 interacts

with the *FOXF1* promoter using conformational chromatin capture assays in BO and or OAC cell lines.

It has been suggested that *FOXF1* is regulated by a distant enhancer. Deletion of overlapping regions mapping 96–257 kb upstream of *FOXF1* (leaving *FOXF1* intact) have been identified in patients with a lethal lung developmental disorder called alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV). The patients shared a 75 kb region which is further away from *FOXF1* than the site of rs9936833. Formalin fixed paraffin embedded (FFPE) material from the lung was available from one patient with a deletion. Levels of FOXF1 in the lung were 70 % lower than comparison samples from individuals without the deletion [87]. The deleted region includes two long nonprotein coding RNA (lncRNA) genes that are expressed in the lung (*LINC01081* and *LINC01082).* siRNAs targeting these lncRNAs reduced FOXF1 expression, suggesting that they are involved in regulation of FOXF1. Multiple transcription factor binding sites, including sites for *GLI1, GLI2,* and *GLI3* which are known to regulate *FOXF1,* were also found in the shared deleted region (SDR). A 0.6 kb portion of the SDR containing a cluster of *GLI* transcription factor binding sites increased transcription from the FOXF1 promoter twofold in reporter assays. Chromosome conformation capture on chip (4C) was also able to show interaction between a small portion of the SDR and the promoter of *FOXF1* in a lung cancer cell line, but not in lymphoblastoid cells, suggesting that this deleted region may regulate tissue-specific expression of FOXF1. The interacting portion of the SDR did not encode the lncRNAs.

While the BO signal does not overlap with the deletions identified in patients with ACD/MPV it is adjacent to it (Fig. 13.3) and this raises the possibility that the protein coding gene desert on 16q24 regulates *FOXF1* expression via discrete elements, each responsible for gene expression in one specific tissue type. The deletion in the ACD/MPV patients is likely to mark the lung-specific enhancer and the BO signal may mark a region involved in esophageal-specific expression. Further functional work is warranted to determine whether this is the case.

TBX5

The G allele of rs2701108 was found to be associated with a reduction in BO risk (Table **13.1**) [38]. This SNP maps 117 kb downstream of *TBX5* (*T-box transcription factor 5*) and 270 kb upstream of *RBM19* (*RNA binding motif 19*). As can be seen in Fig. 13.2c another SNP was more strongly associated with risk of BO following imputation of the region using the 1000 Genomes reference panel. This SNP, rs1920562, maps 131 kb downstream of *TBX5* and 256 kb upstream of *RBM19.* As described in [38] rs1920562 maps to a highly conserved base and a region containing enhancer marks in human embryonic stem cells and lung fibroblasts. rs1920562 is also predicted to alter the binding of IKZF1. It is not clear which gene rs1920562 might be regulating, but *TBX5* is a strong candidate. *TBX5* is involved in cardiac and thoracic development; its deficiency causes thoracic malformations, including

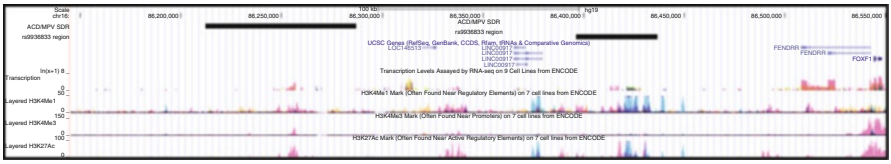

Fig. 13.3 Long range regulation of *FOXF1*. Model of multiple adjacent tissue specific regulatory elements. Data from UCSC genome browser (hg19 assembly) showing ~250 kb upstream of *FOXF1*. This region contains a shared deleted region (SDR) identified in nine patients with a lung development disorder ACD/MPV. Adjacent to this region and closer to *FOXF1* lies the GWAS signal marked by rs9936833. A 40 kb region containing SNPs in high LD with rs9936833 is shown as a custom tract (rs9936833 region). As can be seen the rs9936833 region is marked by histone modifications consistent with active regulatory elements in seven cell lines from ENCODE. A portion of the rs9936833 region also showed some evidence of being transcribed in RNA seq experiments performed by ENCODE on nine cell lines

abnormalities of the diaphragmatic musculature [88], aberrant lung bud and tracheal formation. It has also been shown to be required for cardiac development; mice haploinsufficient for *tbx5* and the obligate hedgehog signaling receptor *smo* show more frequent atrioventricular septal defects than mice haploinsufficient for either gene alone [86]. This suggests some overlap in the gene regulatory networks of Hh signaling and *Tbx5*, at least in the embryological heart.

Smemo et al. [89] have tried to identify *TBX5* enhancers using transgenic mice. They narrowed the enhancer-containing region down to a ~411 kb segment which contains rs2701108 and rs1920562, the most likely functional SNP in LD with the lead genotyped SNP in our study. While rs2701108 and rs1920562 do not map to within the 19 *cis*-regulatory elements tested by Smemo et al. rs1920562 maps to a 261 bp evolutionarily conserved region (ECR) with 77 % identity in opossum (ECR browser, Fig. 13.4). An interesting unanswered question is therefore whether this element has in vivo enhancer activity in humans.

GDF7

As shown in Table **13.1** the A allele of rs3072 was found to be associated with an increased risk of BO [38]. rs3072 maps 7.5 kb downstream of *GDF7* (also known as BMP12) and 6.5 kb downstream of *C2orf43* (Fig. 13.2d). Rs3072 was the most strongly associated SNP in this region on chr2p24 following imputation (Fig. 13.2d). Annotation of this SNP, and SNPs in LD with it, has been described in [38]. Briefly, rs3072 maps to an enhancer region with H3K4Me1 histone marks identified in the lymphoblastoid cell line GM12878 and may alter a GATA binding motif. A SNP in LD with rs3072, rs9306894, was highlighted as likely to affect binding in an analysis of SNPs in this region using RegulomeDB. Data from ENCODE, visualized using UCSC shows that the entire intervening region between *GDF7* and *C2orf43* is transcribed in multiple cell lines and several CEBP binding sites were identified

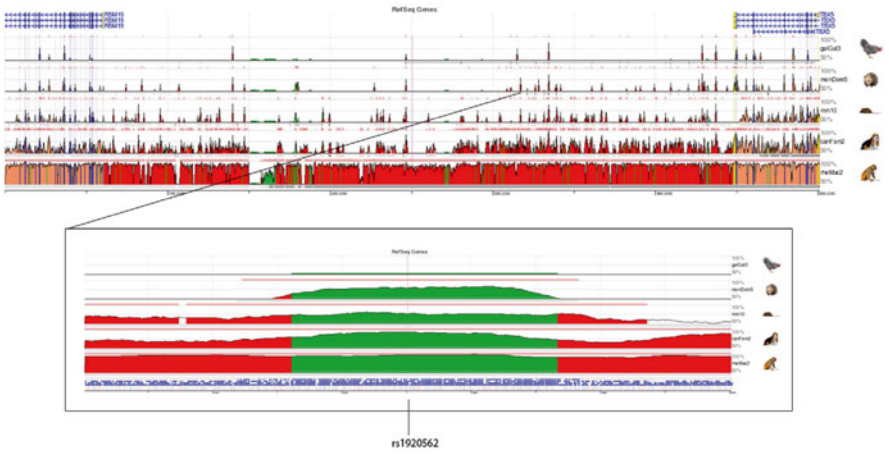

Fig. 13.4 Region containing long range *cis* regulatory elements acting on *Tbx5*. Rs1920562 in LD with a BO GWAS hit maps to a 261 bp evolutionarily conserved region which may also act as an enhancer. ECRs upstream of *TBX5* visualized using the ECR browser (ecrbrowser.dcode.org). rs1920562 maps to a 261 bp ECR conserved back to Possum

in this region by ChIP-seq. CEBP proteins recruit the general transcription coactivator CBP which may recruit Polymerase II to enhancers. Pol2 binding sites were also found by ChIP-seq in the rs3072/rs9306894 region. It has recently been found that many enhancers with neuronal activity produce RNAs (eRNAs) and it has been proposed that transcription of eRNAs generates an open chromatin conformation which enables activation of target genes. *GDF7* is involved in fate specification of cells in the dorsal neural tube as well as playing a role in tendon/ligament development and repair [90, 91]. Perhaps rs3072/rs9306894 is within an eRNA that regulates GDF7.

In a public database of expression data from monocytes, adipose tissue, and lymphoblastoid cell lines, rs9306894 genotype was associated with levels of C2orf43, but not GDF7. Expression data on C2orf43 (but not GDF7) is available from Protein Atlas, showing C2orf43 to be expressed at medium levels in the esophagus. It is therefore possible that *C2orf43* is the target gene in this locus. Little is known of the function of this gene, but a SNP within an intron of *C2orf43* has been shown to be associated with risk of prostate cancer [92].

Despite the lack of an association between rs3072/rs9306894 and *GDF7* expression, this gene may yet be the target of the functional SNP tagged by rs3072; the expression data linking the GWAS signal to C2orf43 was undoubtedly not conducted in the most relevant cell line to BO and esophageal development. *GDF7* encodes the BMP12 protein and the BMP signaling pathway is implicated in the pathophysiology of BO [84]. BMP signaling is involved in the specification of columnar epithelium in the foregut and its expression is restricted by the BMP antagonist Noggin ensuring that the esophageal foregut is not covered with columnar epithelium but squamous epithelium [84].

Esophageal Adenocarcinoma GWAS Hits Are Also in or Near to Genes Involved in Esophageal Development

Two of the hits identified by Levine et al. [40] in a combined analysis of BO and OAC map within or close to transcription factors *BARX1* and *FOXP1,* which have roles in esophageal development. The third hit maps to *CRTC1* (CREB-regulated transcription coactivator), which has been found to be aberrantly activated in cancer. Consistent with the finding that a large proportion of the common genetic variants influencing risk of BO and OAC are shared [93], the GWAS hits identified in a combined analysis of cases show very similar effects when stratified by disease type.

BARX1

The G allele of rs11789015 is protective for BO (Table **13.1**) and the statistical significance of the association was increased by an order of magnitude when AspECT data was combined with BEACON data (Table **13.1** and [38]). rs11789015 maps to an intron of *BARX1* which encodes a homeobox transcription factor. In mice *Barx1* is highly expressed in gastric mesenchyme and is involved in stomach development [94]. In the mouse stomach BARX1 is required for the expression of Wnt antagonists such as secreted frizzled proteins which inhibit Wnt signaling. Barx1 was also found to be expressed in the mesenchymal cells surrounding the murine esophagus, trachea, and bronchi, although not at such high levels as observed in the stomach. *Barx1−/−* mice display a single elongated foregut tube instead of distinct esophageal and tracheal structures. Expression of transcription factors Sox2 and Nkx2.1 is uniform along the tube rather than being confined to the nascent trachea and esophagus, respectively. P63, which is required for differentiation of the stratified squamous epithelium seen in the esophagus, was not detectable in *barx1* mice, suggesting inadequate squamous cell differentiation. Wnt signaling was also detected in the foregut endoderm of *barx1−/−* mice, whereas little to no signal was present in the wild-type mice [94]. This suggests that as in the stomach the role of BARX1 in the dorsal foregut is to restrict Wnt signaling, to allow differentiation of squamous esophageal epithelial cells rather than respiratory epithelium. While Wnt signaling is required early in the development of the foregut, in mice *Barx1* is required to downregulate this signaling for accurate development of the esophagus.

As described in [40] the intronic SNP rs11789015 that marks the GWAS signal on chr9q22 maps to a DNase 1 hypersensitive site and alters a known regulatory motif for *FOXP1*. There are no missense variants in *BARX1* in the 1000 Genomes pilot or release 3 of the HapMap so it is not possible to assess whether the signal rs11789015 is in LD with protein coding variants. If this is the case it must be a variant of large effect given the likelihood that its frequency is <1 %. More likely is the scenario where rs11789015 marks a regulatory region. In vitro and in vivo experiments are required to determine if this is the case and to confirm that *BARX1* is the target gene.

FOXP1

The T allele of rs2687201 mapping to chr3p14 is associated with an increase in risk of BO and OAC (Table **13.1**). This SNP also reached genome-wide significance ($P = 4.61 \times 10^{-8}$) in an analysis restricted to BO cases. The gene closest to the GWAS signal is *FOXP1,* which maps approximately 75 kb away. *FOXP1* is a forkhead transcription factor with DNA-protein and protein-protein binding domains and is involved in esophageal muscle development. Intriguingly, it is the second forkhead protein to be implicated in BO/OAC susceptibility, following rs9936833 which maps approximately 144 kb away from *FOXF1*. Annotation of the SNPs in high LD ($r^2 > 0.8$) with the lead tagging SNP in the locus was performed in [40] and identified rs7626449 as the most likely functional SNP. This SNP maps to a DNase I hypersensitive site which is also marked by H3K4Me1, H3K27Ac, and H3K4Me3 histone modifications associated with regions with promoter or enhancer function. rs7627449 is within *a Nkx2-5b* binding site which is not predicted to be altered in the presence of the variant allele but Sp1 and REV-ErbA binding sites are created by the nonreference A allele (Alibaba2: http://www.generegulation.com/pub/programs/alibaba2/index.html).

FOXP1 is expressed in the muscle compartment and the developing esophageal epithelium in normal mouse embryos, and *Foxf1−/−Foxp1−/+* mice show defects in the muscle surrounding the esophagus with loss of both skeletal and smooth muscle development [95]. *FOXF1* therefore may be involved in the predisposition to hiatus hernia, which in turn predisposes to BO. Furthermore, it has been suggested that *FOXF1* has tumor suppressor function, as it is frequently lost in solid cancers (e.g., colon, stomach) [96]. The expression of *FOXF1* in BO and OAC is not known. However, given that rs2687201 shows a genome-wide significant association in both the combined analysis of BO and OAC and BO-only cases, dysregulation of *FOXF1* might be an early event in esophageal tumorigenesis.

CRTC1

Two intronic SNPs in *CRTC1* (CREB-regulated transcription coactivator 1) were found to be associated with BO/OAC risk at genome-wide significance in a combined analysis [40]. Meta-analysis of data from [40] and discovery phase cases from AsPECT and colorectal cancer study controls [38] provided supportive evidence for one of the two *CRTC1* SNPs, rs10423674 ($P = 0.049$) but not the other rs10419226 ($P = 0.87$) (Table **13.1**). As discussed previously, there was evidence of significant between-study heterogeneity in the meta-analysis of rs10419226 and this SNP was no longer at genome-wide significance in a random effects meta-analysis. The reasons for the heterogeneity are not clear, but further replication of this SNP in an independent dataset seems warranted.

rs10423674 however is genome-wide significant following meta-analysis of both available GWAS studies and so there is still support for the involvement of this locus in BO/OAC risk. rs10423674 maps to an intron of *CRTC1* and has been identified in a separate GWAS study as being associated with age at menarche [97]. *CRTC1* is an excellent candidate gene; it has been shown to have oncogenic potential [98] and its phosphorylation is regulated by tumor suppressor LKB1. *LKB1* expression has been shown to be lost or reduced in human esophageal cell lines and tumor tissue, resulting in activation of CRTC1 signaling, which has been attributed to the increased cell migration and invasion properties of cells where LKB1 was knocked down [99].

eQTL data from lymphoblastoid cells suggests that another gene, *PBX4*, might be the target of the region marked by rs10423674. The rs10423674 locus maps ~1 Mb away from *PBX4* and if the eQTL data is to be believed it may therefore mark a long range enhancer targeting *PBX4*. This is plausible as other enhancer regions have been identified within the introns of nontarget genes, separated by one Mb or more from the gene that they act upon [100]. *PBX4* encodes a homeobox protein belonging to the pre-B cell leukemia family of transcription factors that are involved in embryonic development and cellular differentiation. The zebrafish homolog is required for *shh* expression in the foregut and reduction in pbx4 expression results in an increase in insulin expression in the anterior foregut and underdevelopment of the pharyngeal region [101]. Given the essential role of SHH in human esophageal development it would be interesting to examine whether rs10423674 is an eQTL for *PBX4* or *CRTC1* in a more appropriate cell type than lymphoblastoid cells.

The transcription factors discussed earlier are known to be involved in the development of the thorax, diaphragm, and esophagus and so their dysregulation may contribute to a predisposition to reflux (via mechanical factors such as diaphragmatic sphincter tone, hiatus hernia, and the gastroesophageal angle) and BO and, finally, OAC.

Discussion and Concluding Remarks

What is immediately apparent is that genes near 7 out of the 8 genome-wide significant loci found to be associated with BO/OAC risk have roles in esophageal development. This intriguing finding suggests a model whereby BO and progression to OAC result in part from dysregulation of genes involved in esophageal development. The functional SNPs at each locus remain to be determined and the target genes, while plausible, need confirming through in vitro or in vivo studies. HH signaling, expression of GLI transcription factors, BMP signaling and expression of RA are all increased during the development of BO compared to levels in the normal adult esophagus, and GWAS results add further support to possible therapeutic targeting of transcription factors involved in esophageal development. Indeed, as reviewed in [84], there are clinical trials underway investigating agents inhibiting HH and WNT signaling, either alone or in combination with chemotherapeutics such as Capecitabine (oral 5-FU) in patients with esophageal cancer.

The GWAS studies conducted to date are likely to have only identified the common variants conferring the largest disease risks and far larger sample sizes will be required to identify those variants conferring more modest odds ratios. Indeed, estimates of the proportion of heritability of BO that can be explained by common SNPs on genome-wide tagging SNP arrays suggest that there are many more BO susceptibility variants to be discovered. Two methods to look for evidence of the existence of many common variants influencing risk of BO were described in Su et al. [39]. A sign test was performed which found evidence that an excess of the top 1100 SNPs in discovery phase showed the same direction of effect in the replication data ($P_{\text{uncorrected}} = 2.3 \times 10^{-5}$). A disease score analysis which involved assigning each individual a disease score based upon the number of test alleles they carried before comparing the disease scores of cases and controls also found evidence of a large number of common SNPs of small effect influencing susceptibility to BO ($P_{\text{uncorrected}} = 7.07 \times 10^{-11}$). We subsequently performed Genome-wide Complex Trait Analysis (GCTA) studies to estimate the amount of variation in BE risk explained by all of the autosomal SNPs. This suggested that 9.9 % (SE = 1.2 %) of variation in risk of BO could be explained by common variants. By contrast, Ek et al. [93] performed GCTA using data from BEACON and estimated the proportion of heritability explained by common SNPs at 35 % (SE 6 %) [93]. Both estimates were highly statistically significant ($P < 1 \times 10^{-9}$). The considerable difference between the two estimates may be due to differences in analysis or study design as discussed in [38]. However, even if the contributions of common SNPs to BO heritability would be just 9.9 %, there would still be considerable justification for attempting to identify new BO/OAC susceptibility loci by conducting further GWAS or genome-wide meta-analysis of existing studies. As consortia formed to investigate genetic determinants of other cancers, most notably breast cancer, have been successful in identifying tens of risk loci, it is very likely such studies for BO and OAC would similarly be successful.

Ultimately, if multiple loci could be identified explaining substantial proportions of the variation in BO/OAC susceptibility, these variants could perhaps be used as biomarkers in combination with environmental risk factors, to identify high-risk patients. This would enable targeted screening of individuals at greater risk. As discussed earlier, one of the main clinical challenges of esophageal cancer is its late presentation and consequent bleak prognosis. High risk individuals might benefit from risk reduction through acid suppression and intensive endoscopic surveillance, provided they can be reliably identified.

Currently no variants have been identified that are associated with progression from BO to OAC. Given the findings of Weaver et al. [102] that most of the somatic mutations identified in OAC are also present in BO, it may be challenging to identify the germline determinants of progression from BO to OAC, which occurs in only a minority of BO patients. As the AsPECT trial matures it may be possible to try to identify such genetic variants, but the effects will need to be sizeable to stand any chance of detection given the likely number of BO cases that will have progressed to high dysplasia or OAC. Given the involvement of the immune system in BO/OAC risk it will also be of great interest to see what effect the nonsteroidal

anti-inflammatory drug aspirin has on progression from BO to OAC in AsPECT participants randomized to the treatment arm. However, as more and more patients with BO are identified and enter surveillance programs with prospective registries, comparable GWAS may be able to elucidate the variants and biology involved in the variable progression from BO to OAC.

References

1. Barrett N. Chronic peptic ulcer of the oesophagus and "oesophagitis". Br J Surg. 1950;38:175–82.
2. Watson A, Heading RC, Shepherd NA. Guidelines for the diagnosis and management of Barrett's columnar-lined oesophagus. A report of the Working Party of the British Society of Gastroenterology. 2005. http://www.bsg.org.uk.
3. Cameron AJ, Lomboy CT. Barrett's esophagus: age, prevalence, and extent of columnar epithelium. Gastroenterology. 1992;103(4):1241–5.
4. Ronkainen J, et al. Prevalence of Barrett's esophagus in the general population: an endoscopic study. Gastroenterology. 2005;129(6):1825–31.
5. Cameron AJ, et al. Prevalence of columnar-lined (Barrett's) esophagus. Comparison of population-based clinical and autopsy findings. Gastroenterology. 1990;99(4):918–22.
6. Hvid-Jensen F, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. N Engl J Med. 2011;365(15):1375–83.
7. Wild CP, Hardie LJ. Reflux, Barrett's oesophagus and adenocarcinoma: burning questions. Nat Rev Cancer. 2003;3(9):676–84.
8. Mistry M, et al. Cancer incidence in the United Kingdom: projections to the year 2030. Br J Cancer. 2011;105(11):1795–803.
9. Rutegard M, et al. Population-based esophageal cancer survival after resection without neoadjuvant therapy: an update. Surgery. 2012;152(5):903–10.
10. Davies AR, et al. Factors associated with early recurrence and death after esophagectomy for cancer. J Surg Oncol. 2014;109(5):459–64.
11. Gerson LB, et al. Use of a simple symptom questionnaire to predict Barrett's esophagus in patients with symptoms of gastroesophageal reflux. Am J Gastroenterol. 2001;96(7):2005–12.
12. Sharma P, et al. The development and validation of an endoscopic grading system for Barrett's esophagus: the Prague C & M criteria. Gastroenterology. 2006;131(5):1392–9.
13. Chak A, et al. Familiality in Barrett's esophagus, adenocarcinoma of the esophagus, and adenocarcinoma of the gastroesophageal junction. Cancer Epidemiol Biomarkers Prev. 2006;15(9):1668–73.
14. Verbeek RE, et al. Familial clustering of Barrett's esophagus and esophageal adenocarcinoma in a European cohort. Clin Gastroenterol Hepatol. 2014;12(10):1656–63. e1.
15. Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. Am J Gastroenterol. 1999;94(5):1172–8.
16. Sappati Biyyani RS, et al. Familial trends of inheritance in gastro esophageal reflux disease, Barrett's esophagus and Barrett's adenocarcinoma: 20 families. Dis Esophagus. 2007;20(1):53–7.
17. Mohammed I, et al. Genetic influences in gastro-oesophageal reflux disease: a twin study. Gut. 2003;52(8):1085–9.
18. Sun X, et al. A segregation analysis of Barrett's esophagus and associated adenocarcinomas. Cancer Epidemiol Biomarkers Prev. 2010;19(3):666–74.
19. Bodmer WF. Mutations of the APC (adenomatous polyposis coli) gene in human cancers. Jpn J Cancer Res. 1994;85(6): p. inside front cover.
20. Lindblom A, et al. Genetic mapping of a second locus predisposing to hereditary non-polyposis colon cancer. Nat Genet. 1993;5(3):279–82.

21. Peltomaki P, et al. Genetic mapping of a locus predisposing to human colorectal cancer. Science. 1993;260(5109):810–2.
22. Orloff M, et al. Germline mutations in MSR1, ASCC1, and CTHRC1 in patients with Barrett esophagus and esophageal adenocarcinoma. JAMA. 2011;306(4):410–9.
23. Babar M, et al. Genes of the interleukin-18 pathway are associated with susceptibility to Barrett's esophagus and esophageal adenocarcinoma. Am J Gastroenterol. 2012;107(9):1331–41.
24. Bradbury PA, et al. Matrix metalloproteinase 1, 3 and 12 polymorphisms and esophageal adenocarcinoma risk and prognosis. Carcinogenesis. 2009;30(5):793–8.
25. Casson AG, et al. Cyclin D1 polymorphism (G870A) and risk for esophageal adenocarcinoma. Cancer. 2005;104(4):730–9.
26. di Martino E, et al. The NAD(P)H:quinone oxidoreductase I C609T polymorphism modifies the risk of Barrett esophagus and esophageal adenocarcinoma. Genet Med. 2007;9(6):341–7.
27. Gough MD, et al. Prediction of malignant potential in reflux disease: are cytokine polymorphisms important? Am J Gastroenterol. 2005;100(5):1012–8.
28. Izakovicova Holla L, et al. Haplotypes of the IL-1 gene cluster are associated with gastroesophageal reflux disease and Barrett's esophagus. Hum Immunol. 2013;74(9):1161–9.
29. Kala Z, et al. Polymorphisms of glutathione S-transferase M1, T1 and P1 in patients with reflux esophagitis and Barrett's esophagus. J Hum Genet. 2007;52(6):527–34.
30. MacDonald K, et al. A polymorphic variant of the insulin-like growth factor type I receptor gene modifies risk of obesity for esophageal adenocarcinoma. Cancer Epidemiol. 2009;33(1):37–40.
31. McElholm AR, et al. A population-based study of IGF axis polymorphisms and the esophageal inflammation, metaplasia, adenocarcinoma sequence. Gastroenterology. 2010;139(1): 204–12. e3.
32. Menke V, et al. Functional single-nucleotide polymorphism of epidermal growth factor is associated with the development of Barrett's esophagus and esophageal adenocarcinoma. J Hum Genet. 2012;57(1):26–32.
33. Menke V, et al. NcoI TNF-beta gene polymorphism and TNF expression are associated with an increased risk of developing Barrett's esophagus and esophageal adenocarcinoma. Scand J Gastroenterol. 2012;47(4):378–86.
34. Menke V, et al. Myo9B is associated with an increased risk of Barrett's esophagus and esophageal adenocarcinoma. Scand J Gastroenterol. 2012;47(12):1422–8.
35. Moons LM, et al. A pro-inflammatory genotype predisposes to Barrett's esophagus. Carcinogenesis. 2008;29(5):926–31.
36. van de Winkel A, et al. Expression, localization and polymorphisms of the nuclear receptor PXR in Barrett's esophagus and esophageal adenocarcinoma. BMC Gastroenterol. 2011;11:108.
37. van Lieshout EM, et al. Polymorphic expression of the glutathione S-transferase P1 gene and its susceptibility to Barrett's esophagus and esophageal carcinoma. Cancer Res. 1999;59(3):586–9.
38. Palles C, et al. Polymorphisms near TBX5 and GDF7 are associated with increased risk for Barrett's esophagus. Gastroenterology. 2015;148(2):367–78.
39. Su Z, et al. Common variants at the MHC locus and at chromosome 16q24.1 predispose to Barrett's esophagus. Nat Genet. 2012;44(10):1131–6.
40. Levine DM, et al. A genome-wide association study identifies new susceptibility loci for esophageal adenocarcinoma and Barrett's esophagus. Nat Genet. 2013;45(12):1487–93.
41. Chakravarti A. Population genetics—making sense out of sequence. Nat Genet. 1999;21(1 Suppl):56–60.
42. Lander ES. The new genomics: global views of biology. Science. 1996;274(5287):536–9.
43. Houlston RS, Peto J. The search for low-penetrance cancer susceptibility alleles. Oncogene. 2004;23(38):6471–6.
44. International HapMap Consortium. The International HapMap project. Nature. 2003;426(6968): 789–96.

45. Oliphant A, et al. BeadArray technology: enabling an accurate, cost-effective approach to high-throughput genotyping. Biotechniques 2002(Suppl):56-8, 60-1.
46. Michailidou K, et al. Large-scale genotyping identifies 41 new loci associated with breast cancer risk. Nat Genet. 2013;45(4):353–61. 361e1-2.
47. Spencer CC, et al. Designing genome-wide association studies: sample size, power, imputation, and the choice of genotyping chip. PLoS Genet. 2009;5(5):e1000477.
48. Turner S, et al. Quality control procedures for genome-wide association studies. Curr Protoc Hum Genet. 2011. Chapter 1:Unit1 19.
49. Weale ME. Quality control for genome-wide association studies. Methods Mol Biol. 2010;628:341–72.
50. Winkler TW, et al. Quality control and conduct of genome-wide association meta-analyses. Nat Protoc. 2014;9(5):1192–212.
51. Price AL, et al. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006;38(8):904–9.
52. Altshuler DM, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012;491(7422):56–65.
53. Wessel J, et al. Low-frequency and rare exome chip variants associate with fasting glucose and type 2 diabetes susceptibility. Nat Commun. 2015;6:5897.
54. Zuo X, et al. Whole-exome SNP array identifies 15 new susceptibility loci for psoriasis. Nat Commun. 2015;6:6793.
55. Howie B, Marchini J, Stephens M. Genotype imputation with thousands of genomes. G3 (Bethesda). 2011;1(6):457–70.
56. Whiffin N, et al. Deciphering the genetic architecture of low-penetrance susceptibility to colorectal cancer. Hum Mol Genet. 2013;22(24):5075–82.
57. Kreiner-Moller E, et al. Improving accuracy of rare variant imputation with a two-step imputation approach. Eur J Hum Genet. 2015;23(3):395–400.
58. French JD, et al. Functional variants at the 11q13 risk locus for breast cancer regulate cyclin D1 expression through long-range enhancers. Am J Hum Genet. 2013;92(4):489–503.
59. Amos CI, et al. Genome-wide association study identifies novel loci predisposing to cutaneous melanoma. Hum Mol Genet. 2011;20(24):5012–23.
60. Abnet CC, et al. A shared susceptibility locus in PLCE1 at 10q23 for gastric adenocarcinoma and esophageal squamous cell carcinoma. Nat Genet. 2010;42(9):764–7.
61. Dura P, et al. Barrett associated MHC and FOXF1 variants also increase esophageal carcinoma risk. Int J Cancer. 2013;133(7):1751–5.
62. Lan Q, et al. Genome-wide association analysis identifies new lung cancer susceptibility loci in never-smoking women in Asia. Nat Genet. 2012;44(12):1330–5.
63. Chen D, et al. Genome-wide association study of susceptibility loci for cervical cancer. J Natl Cancer Inst. 2013;105(9):624–33.
64. Lu CC, et al. Nasopharyngeal carcinoma-susceptibility locus is localized to a 132 kb segment containing HLA-A using high-resolution microsatellite mapping. Int J Cancer. 2005;115(5):742–6.
65. Findlay JM, Maynard ND. Pathophysiology and investigation of gastro-oesophageal reflux disease. In: Griffin SM, Raimes SA, Shenfine J, editors. Oesophagogastric surgery. Philadelphia, PA: Saunders/Elsevier; 2013.
66. Souza RF, et al. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. Gastroenterology. 2009;137(5):1776–84.
67. Rieder F, et al. Gastroesophageal reflux disease-associated esophagitis induces endogenous cytokine production leading to motor abnormalities. Gastroenterology. 2007;132(1):154–65.
68. Tomita R, et al. Physiological studies on nitric oxide in the lower esophageal sphincter of patients with reflux esophagitis. Hepatogastroenterology. 2003;50(49):110–4.
69. Cheng L, et al. Inflammation induced changes in arachidonic acid metabolism in cat LES circular muscle. Am J Physiol Gastrointest Liver Physiol. 2005;288(4):G787–97.
70. Eastwood GL, et al. Beneficial effect of indomethacin on acid-induced esophagitis in cats. Dig Dis Sci. 1981;26(7):601–8.

71. Boderg K, Trudgill H. Guidelines for oesophageal manometry and pH monitoring. British Society of Gastroenterology: Guidelines in Gastroenterology; 200.
72. O'Riordan JM, et al. Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am J Gastroenterol. 2005;100(6):1257–64.
73. Tselepis C, et al. Tumour necrosis factor-alpha in Barrett's oesophagus: a potential novel mechanism of action. Oncogene. 2002;21(39):6071–81.
74. Montgomery SB, et al. Transcriptome genetics using second generation sequencing in a Caucasian population. Nature. 2010;464(7289):773–7.
75. Mirvish SS. Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. Cancer Lett. 1995;93(1):17–48.
76. Wu C, et al. Genome-wide association analyses of esophageal squamous cell carcinoma in Chinese identify multiple susceptibility loci and gene-environment interactions. Nat Genet. 2012;44(10):1090.
77. Yoshida A, Hsu LC, Yasunami M. Genetics of human alcohol-metabolizing enzymes. Prog Nucleic Acid Res Mol Biol. 1991;40:255–87.
78. Yin SJ, et al. Polymorphism of human liver alcohol dehydrogenase: identification of ADH2 2-1 and ADH2 2-2 phenotypes in the Japanese by isoelectric focusing. Biochem Genet. 1984;22(1–2):169–80.
79. Fang JL, Vaca CE. Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers. Carcinogenesis. 1997;18(4):627–32.
80. Wang XD, et al. Chronic alcohol intake reduces retinoic acid concentration and enhances AP-1 (c-Jun and c-Fos) expression in rat liver. Hepatology. 1998;28(3):744–50.
81. Osanai M, Lee GH. Increased expression of the retinoic acid-metabolizing enzyme CYP26A1 during the progression of cervical squamous neoplasia and head and neck cancer. BMC Res Notes. 2014;7:697.
82. Freedman ND, et al. Alcohol intake and risk of oesophageal adenocarcinoma: a pooled analysis from the BEACON Consortium. Gut. 2011;60(8):1029–37.
83. Rhinn M, Dolle P. Retinoic acid signalling during development. Development. 2012;139(5):843–58.
84. Pavlov K, et al. Embryological signaling pathways in Barrett's metaplasia development and malignant transformation; mechanisms and therapeutic opportunities. Crit Rev Oncol Hematol. 2014;92(1):25–37.
85. Mahlapuu M, Enerback S, Carlsson P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development. 2001;128(12):2397–406.
86. Hoffmann AD, et al. Foxf genes integrate tbx5 and hedgehog pathways in the second heart field for cardiac septation. PLoS Genet. 2014;10(10):e1004604.
87. Szafranski P, et al. Two deletions overlapping a distant FOXF1 enhancer unravel the role of lncRNA LINC01081 in etiology of alveolar capillary dysplasia with misalignment of pulmonary veins. Am J Med Genet A. 2014;164A(8):2013–9.
88. Arora R, Metzger RJ, Papaioannou VE. Multiple roles and interactions of Tbx4 and Tbx5 in development of the respiratory system. PLoS Genet. 2012;8(8):e1002866.
89. Smemo S, et al. Regulatory variation in a TBX5 enhancer leads to isolated congenital heart disease. Hum Mol Genet. 2012;21(14):3255–63.
90. Berasi SP, et al. Divergent activities of osteogenic BMP2, and tenogenic BMP12 and BMP13 independent of receptor binding affinities. Growth Factors. 2011;29(4):128–39.
91. Lo L, Dormand EL, Anderson DJ. Late-emigrating neural crest cells in the roof plate are restricted to a sensory fate by GDF7. Proc Natl Acad Sci U S A. 2005;102(20):7192–7.
92. Takata R, et al. Genome-wide association study identifies five new susceptibility loci for prostate cancer in the Japanese population. Nat Genet. 2010;42(9):751–4.
93. Ek WE, et al. Germline genetic contributions to risk for esophageal adenocarcinoma, Barrett's esophagus, and gastroesophageal reflux. J Natl Cancer Inst. 2013;105(22):1711–8.

94. Woo J, et al. Barx1-mediated inhibition of Wnt signaling in the mouse thoracic foregut controls tracheo-esophageal septation and epithelial differentiation. PLoS One. 2011;6(7):e22493.
95. Shu W, et al. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. Development. 2007;134(10):1991–2000.
96. Banham AH, et al. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. Cancer Res. 2001;61(24):8820–9.
97. Elks CE, et al. Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. Nat Genet. 2010;42(12):1077–85.
98. Komiya T, et al. Sustained expression of Mect1-Maml2 is essential for tumor cell growth in salivary gland cancers carrying the t(11;19) translocation. Oncogene. 2006;25(45):6128–32.
99. Gu Y, et al. Altered LKB1/CREB-regulated transcription co-activator (CRTC) signaling axis promotes esophageal cancer cell migration and invasion. Oncogene. 2012;31(4):469–79.
100. Lettice LA, et al. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. Hum Mol Genet. 2003;12(14):1725–35.
101. Diiorio P, et al. TALE-family homeodomain proteins regulate endodermal sonic hedgehog expression and pattern the anterior endoderm. Dev Biol. 2007;304(1):221–31.
102. Weaver JM, et al. Ordering of mutations in preinvasive disease stages of esophageal carcinogenesis. Nat Genet. 2014;46(8):837–43.

Part III
Cancer Progression in the Stomach

Chapter 14
Endoluminal Diagnosis of Early Gastric Cancer and Its Precursors: Bridging the Gap Between Endoscopy and Pathology

Noriya Uedo and Kenshi Yao

Early Gastric Cancer and Its Precursors

Definition of Early Gastric Cancer

Although the incidence of gastric cancer has been consistently declining, it remains the fifth most common cancer overall as well as the third leading cause of cancer death worldwide. Japan has one of the highest incidences globally (age-standardized rate of 45.7/100,000 in men and 16.5/100,000 in women) [1]. The Japanese Society of Gastroenterological Endoscopy first defined early gastric cancer (EGC) as adenocarcinoma confined to the mucosa or submucosa, irrespective of lymph node involvement, in 1962 [2]. This definition was based on the fact that this type of gastric cancer shows a particularly good prognosis with 5-year survival rates >95 % after surgery [3]. Lymph node metastasis is found in 10-20 % of these EGC cases, but metastatic lymph nodes are mostly (70 %) restricted to regional nodes (N1) and distant metastasis is rare [4]. Gastrectomy with lymph node dissection thus shows excellent outcome in patients with EGC. When EGC is confined to the mucosa, lymph node involvement is much less common still (≤3 %) [5]. Moreover, intramucosal EGC that fulfills certain endoscopic and histopathological characteristics such as absence of ulceration or scar and differentiated histological type can be a candidate for endoscopic local resection, because lymph node metastasis rate in these

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cases is practically 0 % [6, 7]. Recent advances in diagnostic endoscopy have shown that the percentage of stage I gastric cancers among resected cases exceeds 70 % in leading Japanese cancer referral centers in 2014 [8]. This paper deals with the endoscopic diagnosis of EGC; for endoscopic treatment of EGC, please see the accompanying paper by Oda ("Endoscopic submucosal dissection of early gastric cancer: getting it right").

Histological Criteria

There is an important difference between Japan and the West in the approach to histopathological diagnosis and classification of EGC [9]. In Western countries gastric cancer is diagnosed when neoplastic cells show bona fide invasive growth into the lamina propria of the mucosa [10]. In contrast, Japanese pathologists often refer to intramucosal lesions as malignant, even if these lack clearly demonstrable invasive growth into the lamina propria and would have been classified as (high-grade) dysplasia by Western histopathologists [11]. This discrepancy does not indicate a biological difference of the tumor per se but represents a conceptual difference in approach between Japanese and Western pathologists. However, from a practical viewpoint, a biopsy diagnosis of either high-grade dysplasia or adenocarcinoma is an indication for endoscopic resection in both environments. In the revised Vienna classification [12], EGC according to the Japanese criteria includes all high-grade neoplasia (Category 4.1–4.4) and submucosal invasion by carcinoma (Category 5). In this review, the term EGC therefore includes all intramucosal neoplasia, which, in some cases, would be histopathologically classified as high-grade dysplasia in the West.

Endoscopic Procedures

Preparation

Removal of mucus and bubbles from the mucosal surface before endoscopic inspection is important to improve detection and characterization of mucosal lesions. In Japan, a mixture of water with mucolytic and antifoaming agents (100 ml of water with 20,000 U pronase (Pronase MS, Kaken Pharmaceutical, Tokyo, Japan), 1 g sodium bicarbonate, and 10 ml dimethylpolysiloxane (Gascon, 20 mg/ml, Horii Pharmaceutical Ind., Osaka Japan)) is administered before the procedure [13]. An alternative mixture comprises 100 ml of water mixed with 2 ml of acetylcysteine (200 mg/ml Parvolex, Celltech, UK; or Mucomyst, Bristol-Myers Squibb, USA), and 0.5 ml (40 mg/ml) activated dimethicone (Infacol, Forest Laboratories, Slough, Berkshire, UK) when pronase is not available [14]. When the surface of the detected

lesions is covered with mucous or bubbles, it is rinsed with dimethylpolysiloxane solution before inspection. An anticholinergic agent such as 10–20 mg scopolamine butylbromide (Buscopan, Nippon Boehringer Ingelheim, Tokyo, Japan) or glucagon (1 mg of Glucagon G Novo; Eisai, Tokyo) is given just before inserting the endoscope to inhibit peristalsis.

White Light Endoscopy

Conventional white light endoscopy is a widely available technique. Although dye- or equipment-based image enhanced endoscopy has revolutionized endoscopic diagnosis [15], white light observation still plays a fundamental role in the diagnosis of EGC during routine endoscopy [16]. Adherence to a systematic inspection protocol is recommended to routinely inspect the entire stomach and exclude missing "blind" areas. A basic technique for avoiding blind areas involves adequate air insufflation to expand the gastric wall to separate the folds, rinsing mucus and bubbles from the gastric mucosa by irrigation with antifoaming agent solution, and mapping the entire stomach. Recently, the "systematic screening protocol for the stomach (SSS)" has been proposed to this end (Fig. 14.1a). In this method, images are arranged according to the order of the procedure, and pictures of 4 or 3 quadrant views are taken in either a clockwise or anticlockwise manner [17].

Dye-Based Image-Enhanced Endoscopy (Chromoendoscopy)

Chromoendoscopy facilitates detailed evaluation of morphological characteristics of the surface mucosa which are difficult to evaluate with white light endoscopy (Fig. 14.1b–e) [18]. Indigo carmine chromoendoscopy was developed in the 1970s [19] and has been most commonly used for diagnosis of gastric lesions. Indigo carmine dye accumulates along mucosal crevices and thereby enhances contrast of the mucosal surface topography. A low concentration (0.05 %) is adequate for inspection of gross appearance, while a high concentration (0.2 %) is suitable for evaluation of the microsurface structure under magnifying observation. Mucosal preparation with mucolytic and antifoaming agents or rinsing with antifoaming solution is important in chromoendoscopy [13], because overlying mucous can interfere with proper inspection of the mucosal surface (Fig. 14.2c, d). Methylene blue chromoendoscopy is used for staining of gastric intestinal metaplasia [20].

The dye solution is applied with syringe flushing through the working channel to characterize detected lesions through local application of indigo carmine. A spraying catheter (PW-5 L-1, PW-6P-1, PW-205 V, Olympus Medical Systems, Tokyo, Japan) is useful to improve detection of lesions after pan-gastric application of indigo carmine.

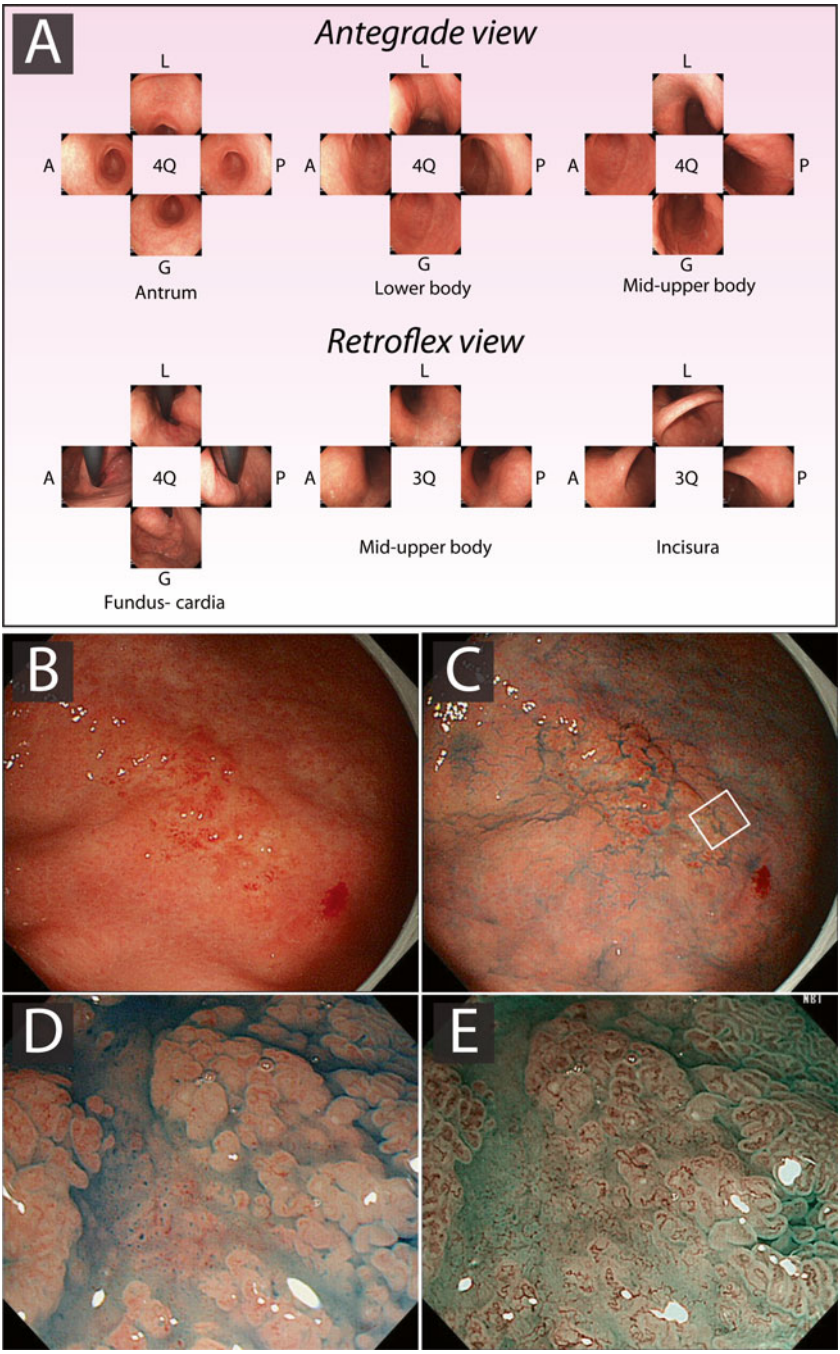

Fig. 14.1 (**a**) Systematic screening protocol for the stomach (SSS, adapted from [17]). (**b**) Type 0-IIc EGC. A localized and irregular redness can be discerned. (**c**) Chromoendoscopy with 0.2 % indigo carmine clearly reveals the irregular surface morphology in overview. (**d**) Magnifying chromoendoscopy reveals the irregular microsurface structure in close-up. (**e**) Magnifying NBI shows chaotic microvascular architecture

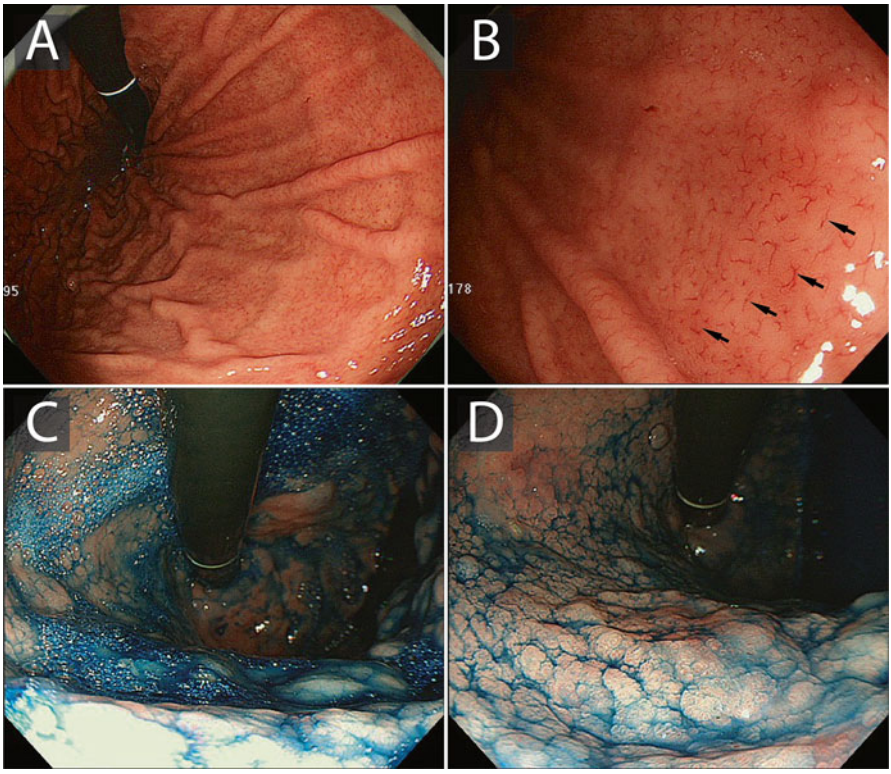

Fig. 14.2 (**a**) *H. pylori*-naïve gastric corpus mucosa. The mucosa looks homogeneously reddish and straight gastric folds are seen. (**b**) In the close-up view, regularly arranged collecting venules are seen (*arrows*). (**c**) When mucous covers the mucosa dye solution does not reach the mucosal surface. (**d**) After irrigation with dimethicone solution

Equipment-Based Image-Enhanced Endoscopy (Narrow Band Imaging, Flexible Spectral Imaging Color Enhancement, iScan)

Chromoendoscopy is a technically demanding and time-consuming procedure. Recently, several equipment-based image-enhancing endoscopy techniques that use optical and/or electrical methods have been developed and introduced in clinical practice [15]. In narrow band imaging (NBI) (Olympus Medical Systems, Co. Inc, Tokyo, Japan) the system emits two specific narrow-banded short wavelength (400–430 and 525–555 nm) from a light source through a special optical filter. Because these wavelengths are well absorbed by hemoglobin, the achieved spectral images contrast vascular architecture and surface structure of the superficial mucosa very well [21]. Flexible spectral imaging color enhancement (FICE) (Fuji Film Co. Ltd, Tokyo, Japan) produces real-time spectral images by computation of white light image signals with a video processor [22]. The iScan system (HOYA group, PENTAX

Medical, Tokyo, Japan) modifies color tones of white light images and enhances characteristics of the targeted lesion as differences in color and brightness [23].

Magnifying Endoscopy

In 1976, Yoshii investigated micromucosal structure of the gastric mucosa in patients with atrophic gastritis with a stereoscope and related this to histological findings [24]. Sakaki et al. later translated the stereoscopic findings to endoscopic findings using fiber-optic zoom endoscopy [25]. After the development of zoom video endoscopy in 1999, several studies on micromucosal findings in normal gastric mucosa, atrophic gastritis, and EGC were published [26–29]. A combination of magnifying endoscopy and chromoendoscopy facilitates the detailed evaluation of microsurface mucosal structure (Fig. 14.1d) [30]. Likewise, a combination of magnifying endoscopy and NBI facilitates the detailed evaluation of mucosal microvascular architecture in addition to microsurface mucosal structure. This technique in effect corresponds to "real-time" histology and increases diagnostic endoscopic biopsy yield for conventional histopathological diagnosis (Fig. 14.1e).

For magnifying observation, a cap is required to stabilize the endoscope and to fix the proper focal distance between the lens and the gastric mucosal surface. A black silicone cap (MAJ-1988, -1989, -1990, Olympus Medical Systems) is to be preferred over a transparent cap (D-201-series, Olympus) for diagnostic purposes. These black caps are softer which minimizes contact bleeding (1) and are also reusable, which makes them cost-effective (2).

Endoscopic Findings

A thorough understanding of endoscopic findings in the nonneoplastic gastric mucosa is important for:

1. Identification of high-risk individuals who may go on to develop gastric cancer.
2. Accurate differentiation of neoplastic lesions from nonneoplastic lesions.
3. Endoscopic visualization of the peripheral tumor boundary for management of lesions.

Normal (*Helicobacter pylori* Naive) Gastric Mucosa

Gastric mucosa has two major types of glands: fundic and pyloric-type glands. On white light imaging, the normal fundic mucosa has smooth gastric folds and, in close-up view, a regular arrangement of collecting venules (RAC) can be

appreciated (Fig. 14.2a, b) [26]. Presence of RAC in corpus mucosa has a sensitivity of 48.0 % and specificity of 95.8 % for the diagnosis of *H. pylori* naïve normal stomach [31].

The normal fundic mucosa has straight glands and round "crypt" openings in the surface epithelium (nb. "crypt" opening is set in apostrophes to denote the difference with bona fide intestinal crypts). Therefore, normal fundic mucosa has regularly arranged round crypt openings that are surrounded by a network of the subepithelial capillaries in magnifying chromoendoscopic and NBI images (Fig. 14.3a–d). Collecting venules can be seen as bluish spider-like vessels. The normal cardiac and pyloric mucosa has oblique and branching glands with continuous, grove-like crypt openings. Cardiac and pyloric mucosa therefore has a "ridged" or villiform epithelium, which encases subepithelial capillaries on magnifying chromoendoscopic and NBI images (Fig. 14.3e–h). Consequently, on magnifying NBI round crypt openings (foveolae) and light brown marginal crypt epithelia (i.e., the superficial epithelium surrounding the foveolar orifice) are surrounded by dark brown subepithelial capillaries in the corpus, corresponding with the regularly arranged tubular structure of the glands in this region of the stomach. Conversely, in the antrum dark brown subepithelial capillaries are surrounded by light brown marginal (i.e., "surface") crypt epithelium [32], corresponding with the ridged or papillary structure of the surface epithelium in this region of the stomach (Fig. 14.4).

Helicobacter pylori-*Associated Chronic Atrophic Gastritis*

Gastric cancer, especially noncardiac type, usually develops in patients with chronic *H. pylori* infection. The surrounding mucosa is therefore often affected by *H. pylori*-associated chronic atrophic gastritis. The *H. pylori*-associated inflammatory cell infiltration, and subsequent mucosal atrophy and intestinal metaplasia alter the microvascular architecture and microsurface structure of the gastric mucosa.

On white light imaging, atrophic mucosa in the gastric corpus looks pale, shows increased visibility of mucosal vessels [33], and a diminishment of normal gastric folds (Fig. 14.5). For a diagnosis of moderate-to-severe histological mucosal atrophy, increased visibility of mucosal vessels has a sensitivity of 48 % and specificity of 87 %, whereas the absence of gastric folds has a sensitivity 67 % and specificity of 85 % [34]. Kimura et al. [33] classified the extent of atrophic mucosa as follows (Fig. 14.6):

- C-1: endoscopic atrophic findings are not visible in the gastric corpus;
- C-2: the atrophic border on the lesser curvature was observed at a lower part of the gastric body;
- C-3: the atrophic border on the lesser curvature was observed at the upper part of the gastric body;
- O-1: the atrophic border is observed between the lesser curvature and the anterior wall;

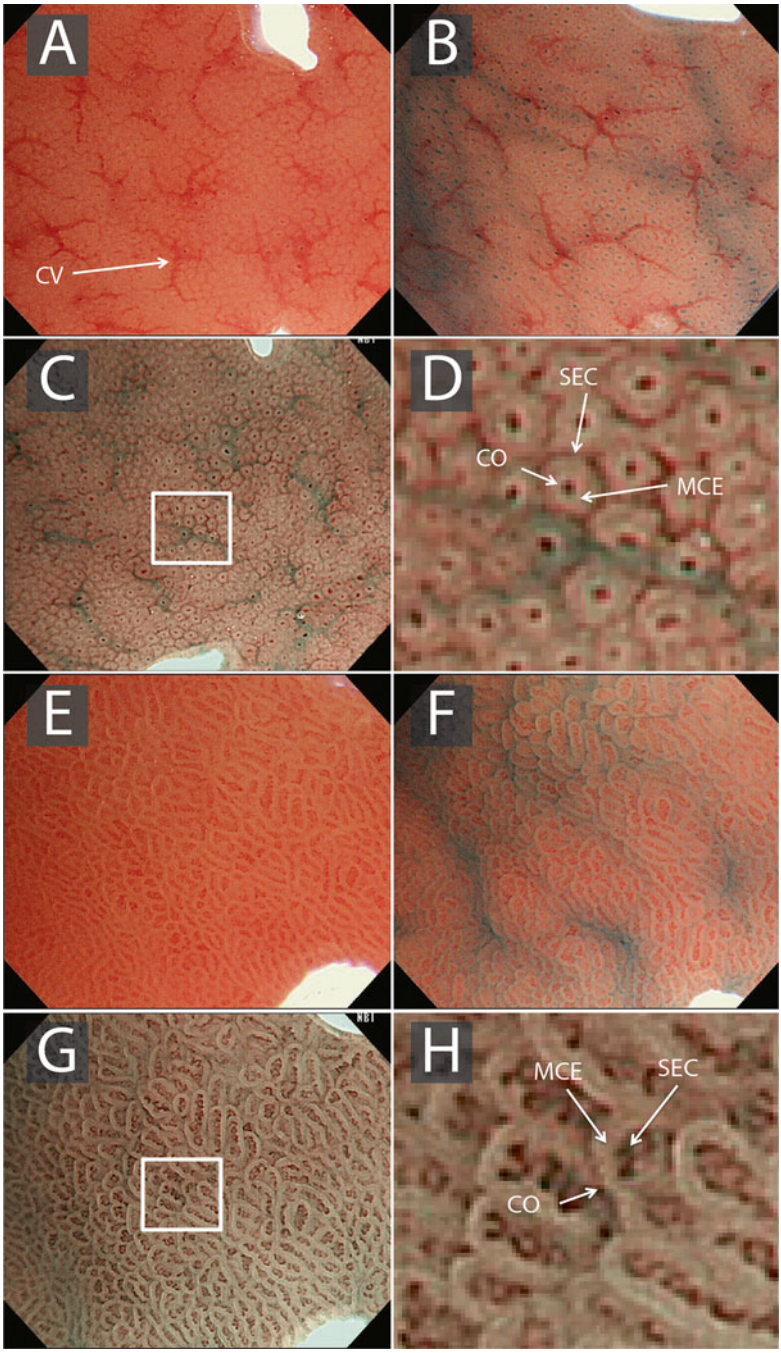

Fig. 14.3 The magnifying endoscopic images of normal corpus and antrum mucosa. (**a**) In magnifying white light image, regularly arranged collecting venules (CV) are seen in the corpus. (**b**) In magnifying chromoendoscopic image, round to oval "crypt" openings can be discerned. (**c**) Magnifying NBI. (**d**) In the enlarged image of (**c**), regularly arranged round crypt openings (CO)

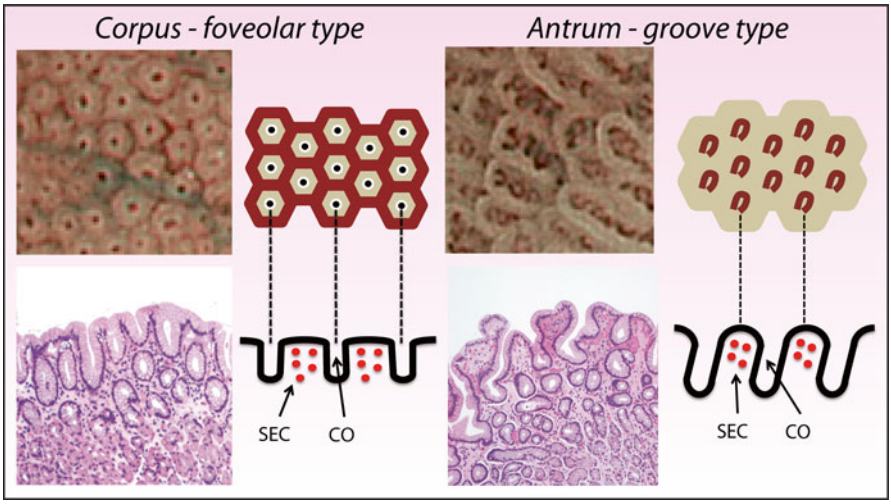

Fig. 14.4 Patterns of magnifying NBI. In the corpus, dark-brownish subepithelial capillaries surround light-brownish marginal crypt epithelia ("foveolar type"), corresponding with the straight tubular gland structure of the superficial mucosa. In the antrum, light-brownish marginal crypt epithelia surround dark-brownish subepithelial capillaries. This corresponds with the ridge/papillary surface structure of the superficial mucosa ("groove type")

- O-2: the atrophic border was observed between the anterior wall and the greater curvature;
- O-3: the atrophic border on the greater curvature was observed proximal to the lower gastric body.

Because the extent of endoscopic atrophy is associated with a relative risk of gastric cancer of 1.7 (95 % C.I. 0.8–3.7) for moderate atrophy and 4.9 (95 % C.I. 2.8–19.2) for severe atrophy [35], endoscopy could be a risk indicator to stratify patients for gastric cancer risk and determine optimal surveillance intervals [36].

Several studies have been carried out on the predictive value of magnifying endoscopic findings in the gastric fundic mucosa for the histological diagnosis of atrophy and intestinal metaplasia [29, 37–39]. In magnifying NBI images, *H. pylori*-positive fundic mucosa without atrophy or intestinal metaplasia shows regularly arranged round crypt openings similar to *H. pylori*-naïve patients, although the normally clearly apparent collecting venule is not seen (Fig. 14.7a). When the mucosa has mild-to-moderate atrophy, the round crypt openings elongate and some become linear (Fig. 14.7b). When atrophy progresses and intestinal metaplasia develops, the mucosal structure changes to the ridged appearance of cardiac/pyloric mucosa,

Fig. 14.3 (continued) and marginal crypt epithelia (MCE) are encompassed by the network of subepithelial capillaries (SEC). (**e**) On magnifying white light imaging, regular ridge-like patterns are seen in the antrum. (**f**) On magnifying chromoendoscopic imaging, micromucosal ridges and grooves of crypt openings can be discerned. (**g**) Magnifying NBI. (**h**) In the enlarged image of panel **g**, coil-like subepithelial capillaries (SEC) are encased in ridge-like marginal crypt epithelia (MCE) that are divided by groove-like crypt openings (CO)

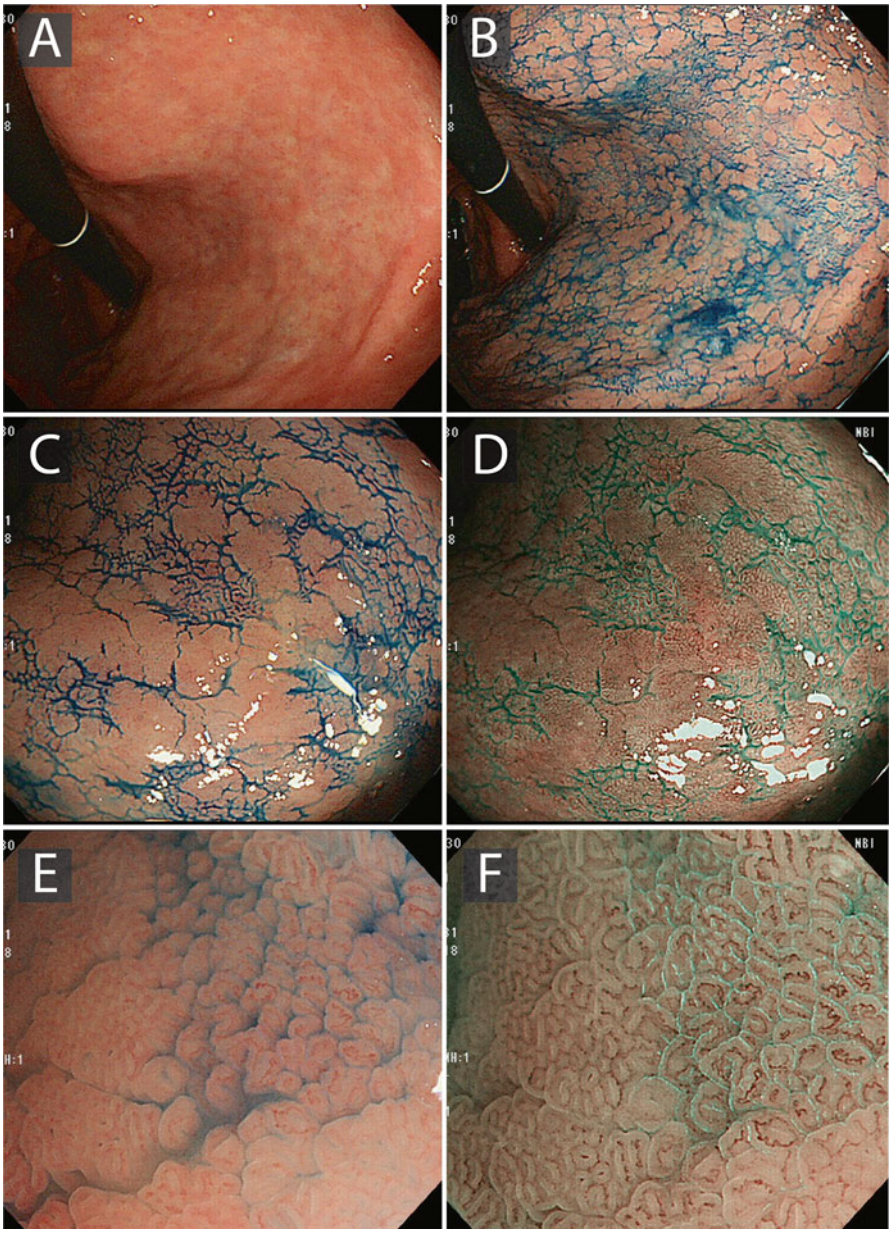

Fig. 14.5 Endoscopic appearance of *H. pylori*-associated chronic atrophic gastritis in the corpus. (**a**) White light imaging reveals that the atrophic mucosa has lost gastric folds and shows a mosaic pattern of pale and reddish mucosal patches. 0.2% Indigo carmine chromoendoscopy on overview (**b**) or with magnification (**c**) reveals that the pale area has a ridged/papillary microsurface structure with dye accumulation, whereas the reddish area sheds the dye because of the flat surface structure. (**d**) Magnifying NBI. (**e**) Mucosa with ridged/papillary microsurface structure shows groove-type mucosa with the light blue crest sign (**f**), suggesting intestinal metaplasia

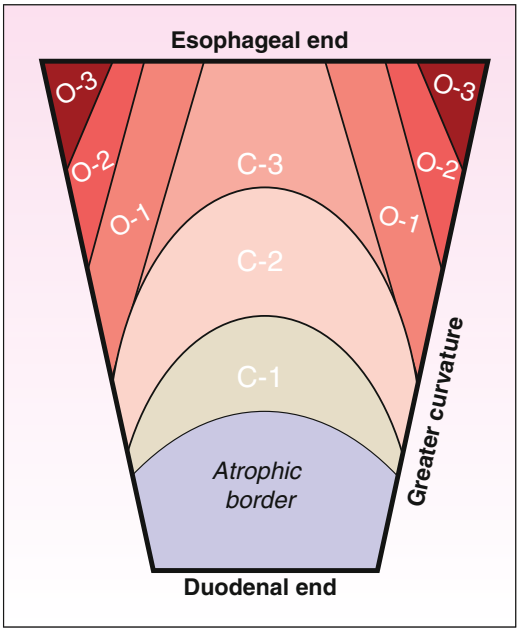

Fig. 14.6 Kimura-Takemoto classification for extent of atrophic gastritis observed by endoscopy (adapted from [33]). Atrophic mucosa is diagnosed by pale color, increased visibility of mucosal vessels, and loss of gastric folds, as explained in the main text

(Fig. 14.7c), or a papillary/villiform appearance similar to intestinal mucosa (Fig. 14.7d). These micromucosal patterns can be classified into two major types: foveolar type and groove type (Fig. 14.7e). The foveolar-type mucosa shows round, oval, or linear crypt openings encompassed by brown subepithelial capillaries. The groove-type mucosa is characterized by ridged, papillary, or villiform mucosal crests divided by continuous grooves of gastric pits, in which the brownish subepithelial capillaries are situated [39]. In other words, the foveolar-type mucosa retains a microstructure similar to the normal fundic mucosa, whereas the groove-type mucosa has transformed into a microstructure more reminiscent of the gastric antrum (so-called endoscopic "antralization") or small intestine ("intestinalization"). Importantly, groove-type mucosa shows a higher degree of atrophy/intestinal metaplasia histopathologically, when compared with foveolar-type mucosa (Fig. 14.7e). Over time multiple small foci of groove-type mucosa develop among foveolar-type mucosa in patients with atrophic/metaplastic mucosa in the gastric corpus. As the disease progresses, the groove-type mucosa expands in a mosaic pattern and eventually replaces confluent areas of the corpus mucosa (Figs. 14.5 and 14.6).

Intestinal Metaplasia

Chronic infection with *H. pylori* provokes molecular alterations in the gastric mucosa and slowly transforms the mucosa into the intestinal phenotype [40]. Endoscopically intestinal metaplasia has a varied appearance. On white light imaging, some intestinal metaplasia appears slightly elevated with whitish patches

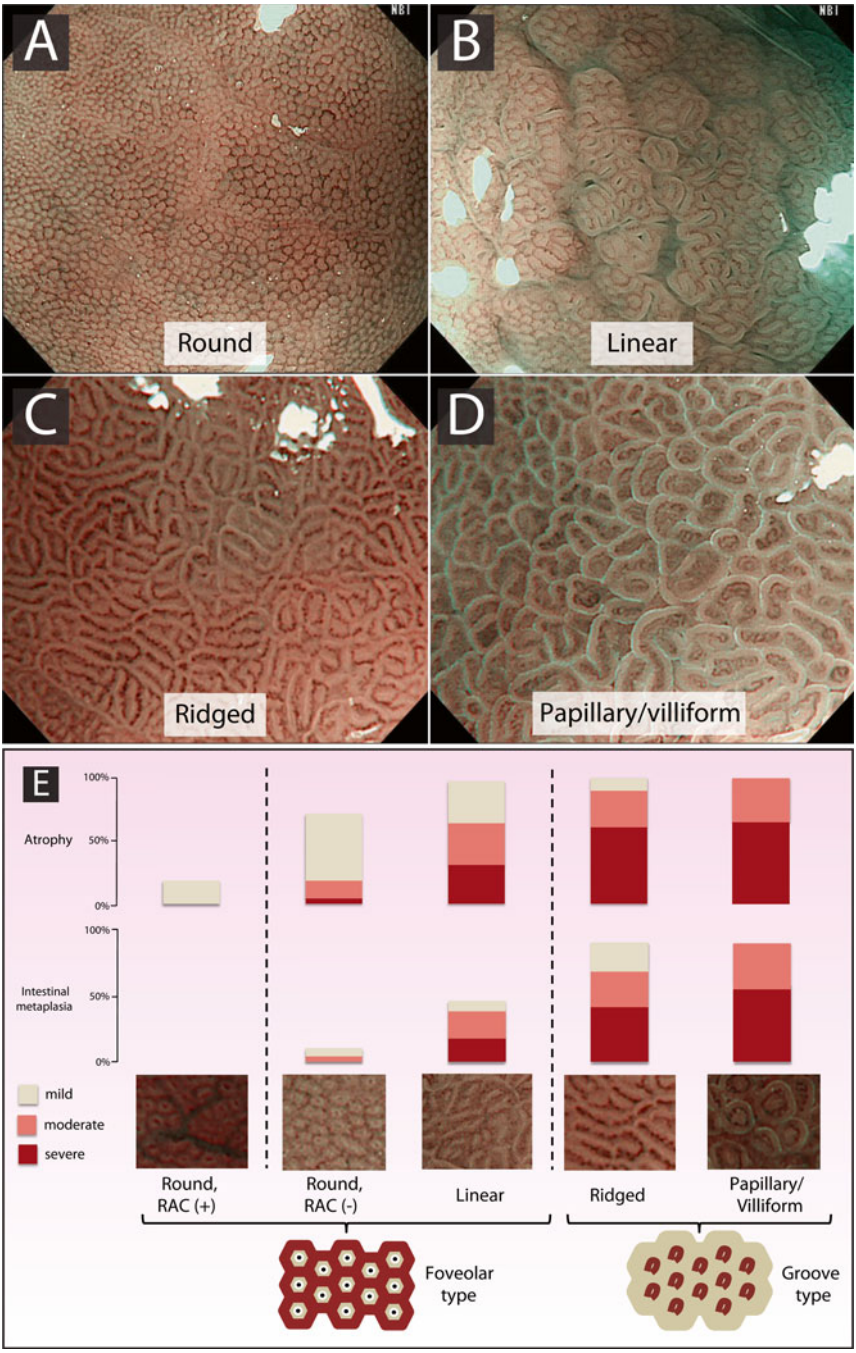

Fig. 14.7 Magnifying NBI of *H. pylori*-associated chronic atrophic gastritis in the gastric corpus. (**a**) Round crypt openings are regularly arranged, but collecting venules are not visualized. (**b**) Elongated (oval to linear) crypt openings are seen. (**c**) Ridged micromucosal structure encases brownish subepithelial capillaries. (**d**) Surface structure looks papillary or villiform and light blue

(Fig. 14.8a). Intestinal metaplasia may also demonstrate mottled reddish depression [41]. NBI further facilitates imaging of intestinal metaplasia by color contrast (Fig. 14.8b). In magnifying NBI images, a fine blue-white line of light is observed on the crests of the epithelial surface/gyri (light blue crest) of intestinal metaplasia (Fig. 14.8c, d) [42]. The "light blue crest" has been thought to derive from the reflection of short wavelength light at the brush border on the surface of intestinal metaplasia. Lipid droplets absorbed by intestinal metaplasia are observed as white opaque substance (WOS) that obscures subepithelial capillaries (Fig. 14.8d) [43]. Moreover, marginal (i.e., "surface") crypt epithelium of intestinal metaplasia looks somewhat "cloudier" than that of nonmetaplastic mucosa [44].

Early Gastric Cancer

Characterization of Detected Lesions

On white light imaging differential diagnosis between cancer and noncancer for suspicious lesions is established according to surface morphology and color. Because superficial type EGC often shows indistinct features, it is important to pay attention to subtle differences in mucosal height and discoloration (faint redness or paleness). For a flat lesion with the same color as the surrounding mucosa, disappearance of the background vascular network or spontaneous bleeding can be a clue. Diagnostic criteria for cancerous lesions are (1) presence of a well-demarcated area and (2) irregularity in surface morphology or color [17, 45]. A presumptive diagnosis of EGC can be established if a lesion fulfills both criteria (flowchart in Fig. 14.9). Indigo carmine chromoendoscopy can further enhance surface morphological features.

The detailed microsurface structure and microvascular architecture of the gastric lesions are best evaluated on magnifying NBI imaging. According to the "vessel plus surface" (VS) classification [46] presence of a clear peripheral demarcation line and irregular microsurface or microvascular patterns are diagnostic criteria for cancerous lesions on magnifying NBI (Fig. 14.9). A diagnosis of EGC is made when a lesion fulfills both criteria [47]. Note that sensitivity of the peripheral demarcation line feature is greater than the irregular microvascular patterns feature, whereas the specificity of the irregular microvascular pattern is greater than that of the demarcation line [48]. In practice, demarcation line features are easier to evaluate than irregular microvascular patterns. Therefore, identification of a demarcation line and subsequent inspection of irregular microvascular patterns in the lesion is

Fig. 14.7 (continued) crests are seen on the epithelial surface. (**e**) Association between histological grade of atrophy/intestinal metaplasia and findings on magnifying NBI of the gastric corpus mucosa. Mucosa with round crypt openings and collecting venules only rarely has atrophy and intestinal metaplasia. The foveolar-type mucosa can demonstrate atrophy, but intestinal metaplasia is rare. The groove-type mucosa has a high rate of atrophy and intestinal metaplasia

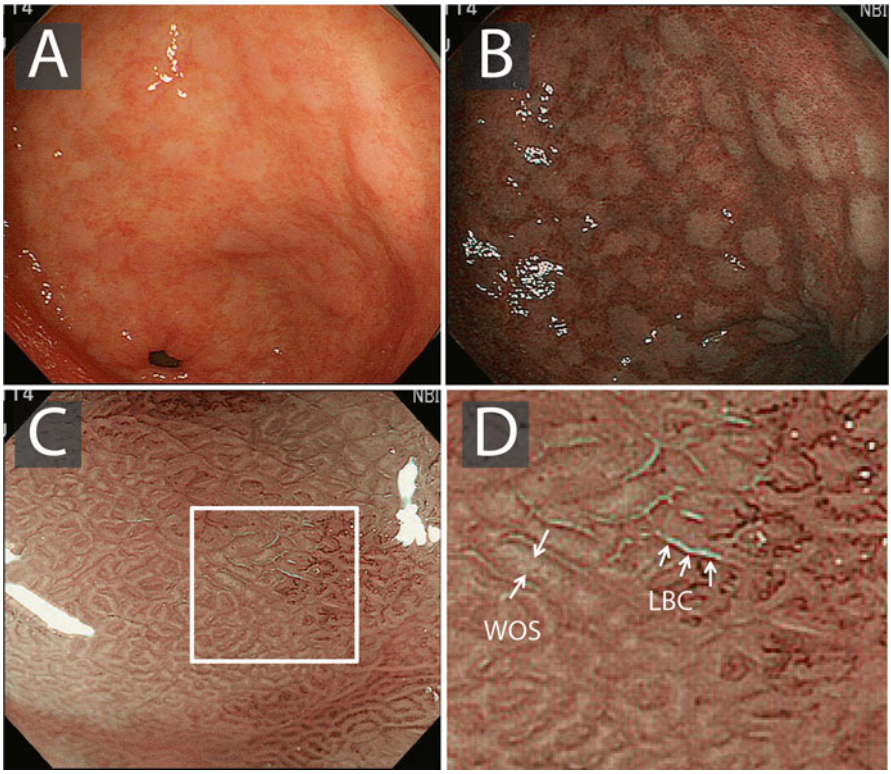

Fig. 14.8 (**a**) Endoscopic findings of intestinal metaplasia. In white light images, intestinal metaplasia demonstrates whitish patches. (**b**) NBI enhances contrast. (**c**) Light blue crests are seen on the surface of the epithelium with magnification and, in some cases, white opaque substance (WOS) is seen in the intervening part of the crypt openings (**d**, corresponds with white square in the **c**)

the preferred strategy for a magnifying NBI diagnosis of EGC. Magnifying NBI provides improved diagnostic accuracy for EGC (sensitivity of 83 % [95 % CI 79–87 %], specificity of 96 % [95–97 %], and AUC of 0.96) in comparison with white light imaging (sensitivity 48 % [39–57 %], specificity 67 % [62–71 %], and AUC 0.62) [49]. Magnifying NBI is especially useful for superficial (gastritis-like) lesions that do not show apparent changes in surface morphology or color.

In some cases of gastric adenoma and superficial elevated-type EGC, the microvascular pattern cannot be evaluated because of the presence of white opaque substance (WOS) that obscures the subepithelial capillaries (Fig. 14.10a, b) [50]. WOS represents intraepithelial lipid droplets that have been absorbed by the neoplastic epithelium expressing intestinal feature [51]. Adenomas have a regular distribution of WOS, whereas EGC is more likely (83 %) to show an irregular distribution of WOS, showing that this feature can further aid endoscopic classification and biopsy yield. So-called white globe appearance (WGA) is a recently reported unique endoscopic marker for EGC (Fig. 14.10c) [52]. Although the sensitivity of WGA is low (21.5 %), its reported specificity is very high (100 %). The WGA is thought to correspond with intraglandular necrotic debris [53] and can therefore also increase biopsy yield.

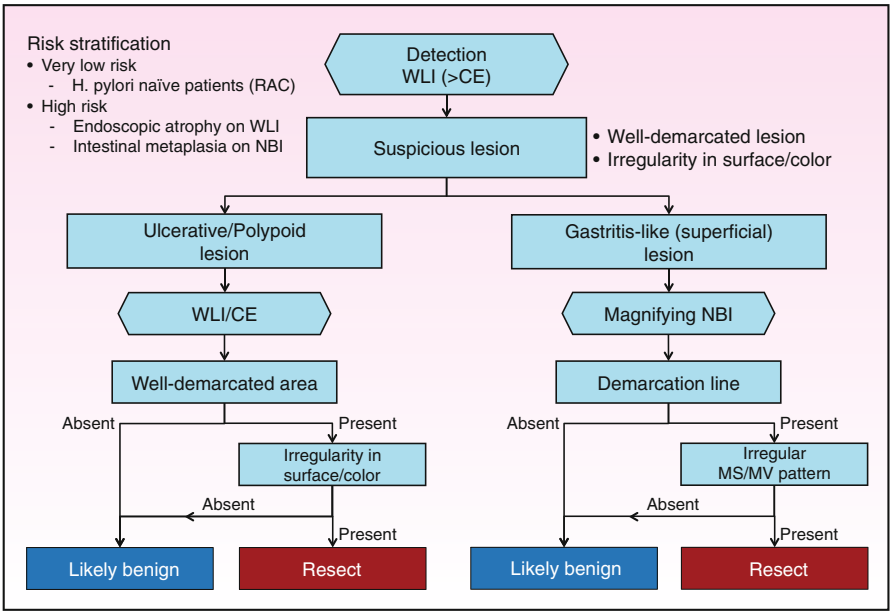

Fig. 14.9 Strategy for endoscopic approach to diagnosis of EGC. Risk of developing gastric cancer is estimated from endoscopic images and suspicious lesions are detected on white light imaging. When lesions are either ulcerative or polypoid, differential diagnosis is made on macroscopic appearance. Chromoendoscopy facilitates evaluation of morphological characteristics of the lesion. For superficial type lesions magnifying NBI is useful. The peripheral demarcation line is identified first, after which irregular patterns are inspected inside the demarcation line (*RAC* regular arrangement of collecting venule, *WLI* white light image, *CE* chromoendoscopy, *NBI* narrow band imaging, adapted from [17])

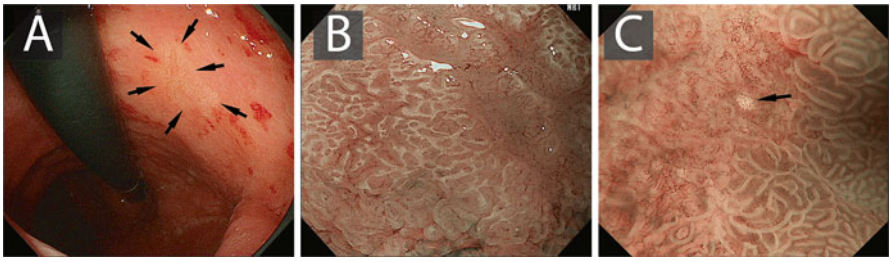

Fig. 14.10 (**a**) Endoscopic images of white opaque substance (WOS). A superficial flat lesion (*arrows*) is observed in the lesser curvature of the lower corpus. (**b**) Irregular distribution of WOS is seen on magnifying NBI. (**c**) Magnifying NBI of white globe appearance (WGA). WGA is observed underneath cancerous gastric epithelium as a small (<1 mm), white lesion with a globular shape (*arrow*)

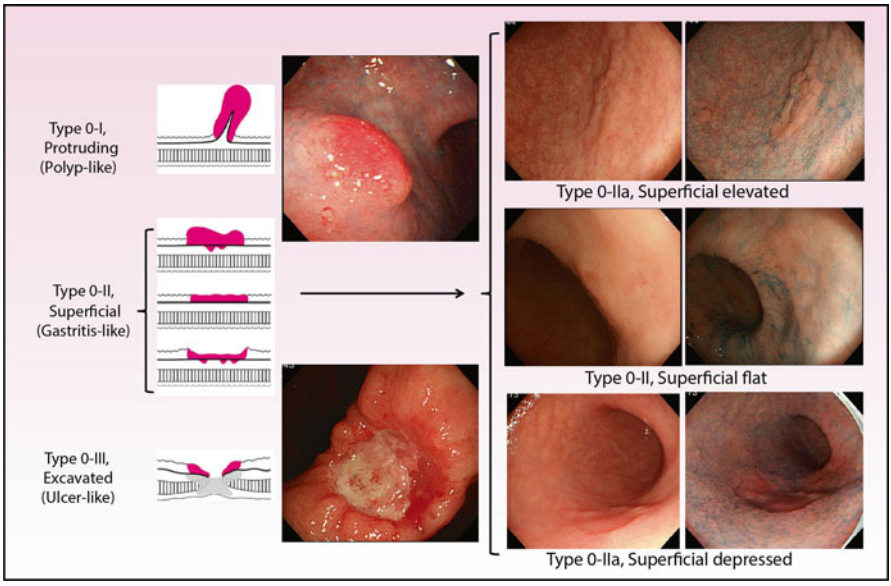

Fig. 14.11 Macroscopic types of early gastric cancer (adapted from [55])

Endoscopic Staging of Early Gastric Cancer

To determine indication of endoscopic resection for EGC histological type, extent (size), depth of tumor, and presence of ulceration or scar, should all be carefully assessed, because the likelihood of concurrent lymph node metastasis is associated with these findings [54].

Macroscopic Type

Macroscopic type of EGC is classified as a Type 0 in the Japanese Gastric Cancer Association Classification (Fig. 14.11) [55] or the Paris classification [56]. Three distinct macroscopic types are protruding (Type 0-I, polyp-like), superficial (Type 0-IIa, gastritis-like), and excavated (Type 0-III, ulcer-like) types. The superficial type is subdivided into superficial elevated (Type 0-IIa), superficial flat (Type 0-IIb), and superficial depressed (Type 0-IIc) types. A tumor with two or more components is recorded in order of the surface area occupied, e.g., 0-IIa + IIc, 0-IIc + IIa, or 0-III + IIc, etc. The majority of EGC contain a depressed (0-IIc; 78 %) or ulcerated (0-III, 2 %) component, whereas the remainder are elevated (0-I, 3 %; 0-IIa, 17 %) with only a very small number of flat lesions (0-IIb, 0.4 %) [55]. The goal is to apply uniform terminology in describing endoscopic appearances to compare rate of submucosal invasion and the risk of lymph node metastases between centers [56].

Histological Type

Gastric cancer demonstrates a wide variation in histological types. It is chiefly divided into two characteristic types according to the presence (intestinal type) or absence (diffuse type) of glandular formation [57]. The Japanese classification of gastric carcinoma [55] subclassifies gastric cancer histopathologically as follows:

- papillary adenocarcinoma
- well and moderately differentiated tubular adenocarcinoma
- mucinous adenocarcinoma
- and poorly differentiated adenocarcinoma and signet ring cell carcinoma (which together correspond with the diffuse type)

It is important to assess histological type at the time of endoscopic diagnosis because this correlates with incidence of lymph node metastases and indication of endoscopic resection. Morphological structure and color of EGC on white light imaging can help predict histopathological type of EGC. Most elevated EGC are of the well to moderately differentiated type. Some gastric adenomas and some superficial elevated-type EGC appear whitish because of the presence of WOS [50]. Among flat or depressed cancers, differentiated-type intramucosal cancers look reddish, whereas undifferentiated type appears whitish because of a difference in hemoglobin content (i.e., vascular density) [58].

Many investigators have described an association between magnifying NBI findings and histological type of EGC. Nakayoshi et al. identified two characteristic microvascular patterns: the fine network and corkscrew patterns in depressed-type EGCs (Fig. 14.12a–h) [59]. The fine network pattern had a sensitivity of 66.1 % and specificity of 96 % for differentiated-type adenocarcinoma, while the corkscrew pattern showed a sensitivity of 86 % and specificity of 96 % for poorly differentiated-type adenocarcinoma. However, 36 % of patients in that study were categorized into the “unclassified” pattern. Yokoyama et al. refined the microvascular classification and rearranged the unclassified pattern as intralobular loop pattern (Fig. 14.12i–m), which is defined as having a papillary microsurface structure, containing looped (coil-like) microvessels [60]. This pattern was subdivided into intralobular loop pattern-1 and intralobular loop pattern-2, depending on the distinctness of the papillary microsurface structure. The intralobular loop pattern-1 was seen only in differentiated-type adenocarcinoma (100 %), whereas the intralobular loop pattern-2 was seen in both differentiated (69 %) and undifferentiated-type adenocarcinoma (21 %). Kobayashi et al. investigated the association between magnifying NBI findings of EGCs and mucin phenotype and found that most tumors with a fine network pattern expressed the intestinal mucin (MUC2, 84.6 %) phenotype, whereas those with the intralobular loop pattern were more likely to express the gastric (MUC5AC and/or MUC6, 61.5 %) or gastrointestinal (30.8 %) mucin phenotype [61]. Kanemitsu et al. recognized a novel mucosal pattern from the intralobular loop pattern, the so-called vessels within epithelial circle (VEC) pattern. This denotes a specific endoscopic pattern in which irregular vessels are completely encircled by

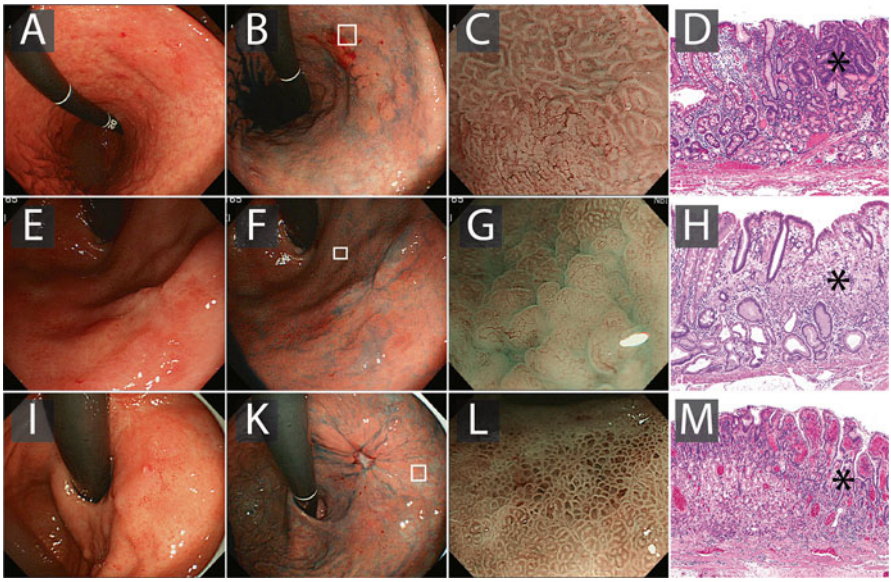

Fig. 14.12 (**a**–**d**) Endoscopic images of a type 0-IIc + IIa EGC. (**a**) A slightly elevated lesion is observed on the lesser curvature of the lower corpus. (**b**) Chromoendoscopy delineates morphological characteristics. (**c**) Magnifying NBI demonstrates the peripheral demarcation line and irregular microvessels that show the fine network pattern (**c**, corresponds with white square in **b**). (**d**) Histopathology shows an intramucosal well-differentiated tubular adenocarcinoma (*asterisk*). (**e**–**h**) Endoscopic images of a type 0-IIb EGC. (**e**) A small pale flat lesion is seen on the anterior wall of the upper corpus. (**f**) Chromoendoscopy contrasts pale color of the lesion. (**g**) Magnifying NBI shows the demarcation line and irregular microvessels with a distinct corkscrew pattern (**g**, corresponds with white square in **f**). (**h**) Histopathology shows a signet-ring cell carcinoma (*asterisk*). (**i**–**m**) Endoscopic images of a type 0-IIa EGC. (**i**) A slightly elevated lesion is seen in the subcardia. (**k**) Chromoendoscopy contrasts morphological characteristics of the lesion. (**l**) Magnifying NBI shows the demarcation line and irregular microsurface patterns (micro-papillae) (**l**, correspond with white square in **l**). (**m**) Histopathology shows an intramucosal moderately differentiated tubular adenocarcinoma with papillary surface structure (*asterisk*)

the curved surface epithelium [62]. The VEC pattern was associated with a papillary histopathological architecture of the surface epithelium and demonstrated a higher incidence of a coexisting poorly differentiated cancer component and tumor submucosal invasion than the VEC-negative pattern. Kanesaka et al. noticed that the "absent microsurface pattern" was indicative of undifferentiated-type adenocarcinoma (sensitivity 92.9 %, specificity 79.7 %) [63]. However, Okada et al. have suggested that the microsurface structure was preserved in 31 % of cases of poorly differentiated adenocarcinoma, because the poorly differentiated cancer cells existed only in the middle to deep layer of the lamina propria [64]. External validation of these endoscopic classifications in relation to clinically relevant outcomes is eagerly awaited. With regards to the relationship between microsurface structure and microvascular architecture, the fine network pattern of EGC corresponds to the foveolar type of background mucosa and the intralobular loop pattern of EGC corresponds to the groove type of background mucosa.

Tumor Extent

Horizontal tumor extent is evaluated according to the presence or absence of characteristic findings of EGC (i.e., difference of mucosal surface morphology and color) in the surrounding mucosa. It is first estimated by white light image, but subsequent image-enhanced endoscopy such as chromoendoscopy and/or magnifying NBI is necessary to achieve a more accurate diagnosis. The peripheral delineation rate on white light imaging for EGC is reported as 72.2 % [65]. Nagahama et al. suggested that 81.1 % of EGC were successfully delineated with indigo carmine endoscopy. For those with unclear margin on indigo carmine endoscopy, subsequent magnifying NBI increased the successful peripheral delineation rate to 94.0 % [66]. For EGCs that showed unclear margins in both chromoendoscopy and magnifying NBI, 59 % were of the well and moderately differentiated type and 41 % were of the poorly differentiated type. Poorly differentiated EGC sometimes expands below the surface epithelium and the true tumor extent is therefore not reflected in surface mucosal appearance. Mapping biopsies from the surrounding mucosa can be required to determine the exact tumor boundary. Magnifying NBI provides useful information for diagnosis of tumor extent, but only a small area of mucosa can be inspected. For this reason, chromoendoscopy remains essential in evaluating the gross appearance of lesions and proper diagnosis of tumor extent.

Tumor Depth

In most high volume centers the vertical depth of the EGC is estimated mainly from morphological characteristics on white light imaging and chromoendoscopy. Endoscopic ultrasound (EUS) is performed in case of inconclusive findings. Large (>30 mm) tumors with pronounced redness, uneven surface features, and marginal elevation predict deep submucosal invasion with a sensitivity of 57.3 % [49.4–65.3 %], specificity of 86.2 % [49.4–65.3 %], and accuracy of 81.1 % [78.5–83.8 %], respectively [67]. Choi et al. demonstrated diagnostic performance of conventional endoscopy by experienced endoscopists for distinguishing T1 mucosal (T1m) from submucosal (T1sm) cancer (accuracy of 78.0 %, sensitivity of 85.5 %, and specificity of 73.9 %) [68]. The same author suggested in a second study that accuracy of conventional endoscopy outperformed EUS diagnosis (73.7 vs. 67.4 % for overall accuracy, $p < 0.001$) [69].

For EUS diagnosis, standard echoendoscope (GIF-UMQ200; Olympus Medical Systems) or a miniprobe (UM-2R, UM-3R; Olympus Medical Systems) is used. 20 MHz is recommended for assessment of tumor depth and 7.5 MHz is used to observe extramural lymph nodes. A meta-analysis suggested that EUS could differentiate T1 mucosal (T1m) from submucosal (T1sm) tumors with a sensitivity of 83 % and specificity of 79 % [70]. Compared with the diagnostic accuracy of EUS in discriminating T1 tumors from more advanced lesions, EUS is less useful in discriminating T1a tumors from T1b tumors. Magnifying NBI has not proven to have

significant advantage for diagnosis of tumor depth [71]. It is therefore clear that every diagnostic imaging method has specific limitations in assessing tumor depth. Histopathological analysis of endoscopically resected specimens is the gold standard reference for tumor staging. Therefore, when there is a possibility of intramucosal carcinoma without definitive evidence of massive submucosal invasion, ESD is usually carried out after explaining the possibility of additional surgery to the patient.

Summary

- Conventional white light endoscopy still plays an important role in the diagnosis of EGC during routine endoscopy. Chromoendoscopy enhances contrast and morphological characteristics of mucosal lesions which are difficult to evaluate with white light endoscopy. NBI enhances vascular architecture as well as surface structure of the superficial mucosa. With magnification, NBI facilitates detailed evaluation of microvascular architecture and microsurface structure enabling "real-time" histopathology.
- Major endoscopic characteristics of gastric epithelial neoplasia are (1) irregular (uneven, asymmetric, or heterogeneous) surface morphology and color; and (2) adherence of neoplastic cells that represents as a demarcation of the lesion.
- Positive diagnostic findings of EGC are a well-demarcated area and irregularity in surface morphology or color on white light imaging and indigo carmine chromoendoscopy. In magnifying NBI, a sharp peripheral demarcation line and irregular microvescular pattern and/or irregular microsurface pattern are positive diagnostic criteria for EGC.
- Histological type of EGC can be predicted from the appearance of the irregular microvascular pattern on magnifying NBI: a fine network pattern and intralobular loop pattern with distinct microsurface structure are indicative of the differentiated type; an intralobular loop pattern with indistinct microsurface structure and corkscrew pattern suggests the poorly differentiated-type adenocarcinoma.
- Horizontal tumor extent of EGC is first evaluated on white light imaging and chromoendoscopy (mucosal color and height). Magnifying NBI improves diagnostic accuracy of white light imaging and chromoendoscopy.
- Depth of tumor invasion is assessed on white light imaging according to gross morphological characteristics. EUS is performed in case of inconclusive findings.

References

1. GLOBOCAN2012 http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.
2. Murakami T. Pathomorphological diagnosis. Definition and gross classification of early gastric cancer. Gann Monogr Cancer Res. 1971;11:53–5.
3. Yamazaki H, Oshima A, Murakami R, Endoh S, Ubukata T. A long-term follow-up study of patients with gastric cancer detected by mass screening. Cancer. 1989;63:613–7.

4. Everett AM, Axon ATR. Early gastric cancer in Europe. Gut. 1997;41:142–50.
5. Sano T, Kobori O, Muto T. Lymph node metastasis from early gastric cancer: endoscopic resection of tumour. Br J Surg. 1992;79:241–4.
6. Gotoda T, Yanagisawa A, Sasako M, et al. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. Gastric Cancer. 2000;3:219–25.
7. Hirasawa T, Gotoda T, Miyata S, et al. Incidence of lymph node metastasis and the feasibility of endoscopic resection for undifferentiated-type early gastric cancer. Gastric Cancer. 2009;12:148–52.
8. Cancer statistics in Japan, 2014 http://ganjoho.jp/data/professional/statistics/backnumber/2014/cancer_statistics_2014.pdf.
9. Schlemper RJ, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, Stolte M, Watanabe H, Takahashi H, Fujita R. Differences in diagnostic criteria for gastric carcinoma between Japanese and western pathologists. Lancet. 1997;349:1725–9.
10. Lansdown M, Quirke P, Dixon MF, Axon ATR, Johnston D. High grade dysplasia of the gastric mucosa: a marker for gastric carcinoma. Gut. 1990;31:977–83.
11. Lewin KJ, Riddell RH, Weinstein WM. Gastrointestinal pathology and its clinical implications. New York: Igaku-Shoin; 1992.
12. Dixon MF. Gastrointestinal epithelial neoplasia: Vienna revisited. Gut. 2002;51:130–1.
13. Fujii T, Iishi H, Tatsuta M, Hirasawa R, Uedo N, Hifumi K, Omori M. Effectiveness of premedication with pronase for improving visibility during gastroendoscopy: a randomized controlled trial. Gastrointest Endosc. 1998;47:382–7.
14. Chang CC, Chen SH, Lin CP, Hsieh CR, Lou HY, Suk FM, Pan S, Wu MS, Chen JN, Chen YF. Premedication with pronase or N-acetylcysteine improves visibility during gastroendoscopy: an endoscopist-blinded, prospective, randomized study. World J Gastroenterol. 2007;13:444–7.
15. Kaltenbach T, Sano Y, Friedland S, Soetikno R, American Gastroenterological Association. American Gastroenterological Association (AGA) Institute technology assessment on image-enhanced endoscopy. Gastroenterology. 2008;134:327–40.
16. Uedo N, Fujishiro M, Goda K, Hirasawa D, Kawahara Y, Lee JH, Miyahara R, Morita Y, Singh R, Takeuchi M, Wang S, Yao T. Role of narrow band imaging for diagnosis of early-stage esophagogastric cancer: current consensus of experienced endoscopists in Asia-Pacific region. Dig Endosc. 2011;23 Suppl 1:58–71.
17. Yao K. The endoscopic diagnosis of early gastric cancer. Ann Gastroenterol. 2013;26:11–22.
18. ASGE Technology Committee, Wong Kee Song LM, Adler DG, Chand B, Conway JD, Croffie JM, Disario JA, Mishkin DS, Shah RJ, Somogyi L, Tierney WM, Petersen BT. Chromoendoscopy. Gastrointest Endosc. 2007;66:639–49.
19. Ida K, Hashimoto Y, Takeda S, Murakami K, Kawai K. Endoscopic diagnosis of gastric cancer with dye scattering. Am J Gastroenterol. 1975;63:316–20.
20. Dinis-Ribeiro M, da Costa-Pereira A, Lopes C, et al. Magnification chromoendoscopy for the diagnosis of gastric intestinal metaplasia and dysplasia. Gastrointest Endosc. 2003;57:498–504.
21. Gono K, Obi T, Yamaguchi M, Ohyama N, Machida H, Sano Y, Yoshida S, Hamamoto Y, Endo T. Appearance of enhanced tissue features in narrow-band endoscopic imaging. J Biomed Opt. 2004;9:568–77.
22. Osawa H, Yoshizawa M, Yamamoto H, et al. Optimal band imaging system can facilitate detection of changes in depressed type early gastric cancer. Gastrointest Endosc. 2008;67:226–34.
23. Kodashima S, Fujishiro M. Novel image-enhanced endoscopy with i-Scan technology. World J Gastroenterol. 2010;16:1043–9.
24. Yoshii T. Comparative study on chronic gastritis with dissecting microscopic findings of stained stomach and its histological findings: application to endoscopic diagnosis. Progr Dig Endosc. 1976;9:49–53. in Japanese.

25. Sakaki N, Iida Y, Okazaki Y, Kawamura S, Takemoto T. Magnifying endoscopic observation of the gastric mucosa, particularly in patients with atrophic gastritis. Endoscopy. 1978;10:269–74.
26. Yagi K, Nakamura A, Sekine A. Characteristic endoscopic and magnified endoscopic findings in the normal stomach without Helicobacter pylori infection. J Gastroenterol Hepatol. 2002;17:39–45.
27. Yao K, Oishi T, Matsui T, Yao T, Iwashita A. Novel magnified endoscopic findings of microvascular architecture in intramucosal gastric cancer. Gastrointest Endosc. 2002;56:279–84.
28. Nakagawa S, Kato M, Shimizu Y, Nakagawa M, Yamamoto J, Luis PA, Kodaira J, Kawarasaki M, Takeda H, Sugiyama T, Asaka M. Relationship between histopathologic gastritis and mucosal microvascularity: observations with magnifying endoscopy. Gastrointest Endosc. 2003;58:71–5.
29. Anagnostopoulos GK, Yao K, Kaye P, Fogden E, Fortun P, Shonde A, Foley S, Sunil S, Atherton JJ, Hawkey C, Ragunath K. High-resolution magnification endoscopy can reliably identify normal gastric mucosa, Helicobacter pylori-associated gastritis, and gastric atrophy. Endoscopy. 2007;39:202–7.
30. Ohnita K, Isomoto H, Shikuwa S, Yamaguchi N, Nakayama T, Nishiyama H, Okamoto K, Fukuda E, Takeshima F, Hayashi T, Kohno S, Nakao K. Magnifying chromoendoscopic findings of early gastric cancer and gastric adenoma. Dig Dis Sci. 2011;56:2715–22.
31. Kato T, Yagi N, Kamada T, Shimbo T, Watanabe H, Ida K, Study Group for Establishing Endoscopic Diagnosis of Chronic Gastritis. Diagnosis of Helicobacter pylori infection in gastric mucosa by endoscopic features: a multicenter prospective study. Dig Endosc. 2013;25:508–18.
32. Bansal A, Ulusarac O, Mathur S, Sharma P. Correlation between narrow band imaging and non-neoplastic gastric pathology: a pilot feasibility trial. Gastrointest Endosc. 2008;67(2):210–6.
33. Kimura K, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. Endoscopy. 1969;1:87–97.
34. Redeen S, Petersson F, Jonsson KA, Borch K. Relationship of gastroscopic features to histological findings in gastritis and Helicobacter pylori infection in a general population sample. Endoscopy. 2003;35:946–50.
35. Uemura N, Okamoto S, Yamamoto S, et al. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
36. Uedo N. Do we need multiple biopsies for assessing gastric cancer risk? Dig Dis Sci. 2011;56:926–8.
37. Tahara T, Shibata T, Nakamura M, et al. Gastric mucosal pattern by using magnifying narrow-band imaging endoscopy clearly distinguishes histological and serological severity of chronic gastritis. Gastrointest Endosc. 2009;70:246–53.
38. Kawamura M, Abe S, Oikawa K, Terai S, Saito M, Shibuya D, Kato K, Shimada T, Uedo N, Masuda T. Topographic differences in gastric micromucosal patterns observed by magnifying endoscopy with narrow band imaging. J Gastroenterol Hepatol. 2011;26:477–83.
39. Kanzaki H, Uedo N, Ishihara R, Nagai K, Matsui F, Ohta T, Hanafusa M, Hanaoka N, Takeuchi Y, Higashino K, Iishi H, Tomita Y, Tatsuta M, Yamamoto K. Comprehensive investigation of areae gastricae pattern in gastric corpus using magnifying narrow band imaging endoscopy in patients with chronic atrophic fundic gastritis. Helicobacter. 2012;17:224–31.
40. Busuttil RA, Boussioutas A. Intestinal metaplasia: a premalignant lesion involved in gastric carcinogenesis. J Gastroenterol Hepatol. 2009;24:193–201.
41. Nagata N, Shimbo T, Akiyama J, et al. Predictability of gastric intestinal metaplasia by mottled patchy erythema seen on endoscopy. Gastroenterol Res. 2011;4:203–9.
42. Uedo N, Ishihara R, Iishi H, et al. A new method of diagnosing gastric intestinal metaplasia: narrow-band imaging with magnifying endoscopy. Endoscopy. 2006;38:819–24.
43. Yao K, Iwashita A, Tanabe H, et al. Author's reply to Letter to the Editor, "White opaque substance" and "light blue crest" within gastric flat tumors or intestinal metaplasia: same or different signs? Gastrointest Endosc. 2009;70(402):402–3.

44. An JK, Song GA, Kim GH, et al. Marginal turbid band and light blue crest, signs observed in magnifying narrow-band imaging endoscopy, are indicative of gastric intestinal metaplasia. BMC Gastroenterol. 2012;12:169.
45. Yao K, Iwashita A, Yao T, Furukawa H, Furukawa K, Takemura S, Matsui T. Quantitative method to characterize electronic endoscopic color images of early gastric carcinomas, using index of hemoglobin. Gastraenterol Endosc. 1997;39:2253–63. Japanese with English abstract.
46. Yao K, Anagnostopoulos GK, Ragunath K. Magnifying endoscopy for diagnosing and delineating early gastric cancer. Endoscopy. 2009;4:462–7.
47. Ezoe Y, Muto M, Uedo N, et al. Magnifying narrowband imaging is more accurate than conventional white-light imaging in diagnosis of gastric mucosal cancer. Gastroenterology. 2011;141:2017–25.
48. Yamada S, Doyama H, Yao K, et al. An efficient diagnostic strategy for small, depressed early gastric cancer with magnifying narrow-band imaging: a post-hoc analysis of a prospective randomized controlled trial. Gastrointest Endosc. 2014;79:55–63.
49. Zhang Q, Wang F, Chen ZY, Wang Z, Zhi FC, Liu S, Bai Y. Comparison of the diagnostic efficacy of white light endoscopy and magnifying endoscopy with narrow band imaging for early gastric cancer: a meta-analysis. Gastric Cancer. 2015 Apr 29. [Epub ahead of print].
50. Yao K, Iwashita A, Tanabe H, et al. White opaque substance within superficial elevated gastric neoplasia as visualized by magnification endoscopy with narrow-band imaging: a new optical sign for differentiating between adenoma and carcinoma. Gastrointest Endosc. 2008;68:574–80.
51. Yao K, Iwashita A, Nambu M, et al. Nature of white opaque substance in gastric epithelial neoplasia as visualized by magnifying endoscopy with narrow-band imaging. Dig Endosc. 2012;24:419–25.
52. Doyama H, Yoshida N, Tsuyama S, Ota R, Takeda Y, Nakanishi H, Tsuji K, Tominaga K, Tsuji S, Takemura K, Yamada S, Katayanagi K, Kurumaya H, Iwashita A, Yao K. The "white globe appearance" (WGA): a novel marker for a correct diagnosis of early gastric cancer by magnifying endoscopy with narrow-band imaging (M-NBI). Endosc Int Open. 2015;3:E120–4.
53. Watanabe Y, Shimizu M, Itoh T, Nagashima K. Intraglandular necrotic debris in gastric biopsy and surgical specimens. Ann Diagn Pathol. 2001;5:141–7.
54. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). Gastric Cancer. 2011;14:113–23.
55. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer. 2011;14:101–12.
56. Participants in the Paris Workshop. The Paris endoscopic classification of superficial neoplastic lesions: esophagus, stomach, and colon: November 30 to December 1, 2002. Gastrointest Endosc. 2003;58(6 Suppl):S3–43.
57. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. Acta Pathol Microbiol Scand. 1965;64:31–49.
58. Yao K, Yao T, Matsui T, Iwashita A, Oishi T. Hemoglobin content in intramucosal gastric carcinoma as a marker of histologic differentiation: a clinical application of quantitative electronic endoscopy. Gastrointest Endosc. 2000;52:241–5.
59. Nakayoshi T, Tajiri H, Matsuda K, Kaise M, Ikegami M, Sasaki H. Magnifying endoscopy combined with narrow band imaging system for early gastric cancer: correlation of vascular pattern with histopathology (including video). Endoscopy. 2004;36:1080–4.
60. Yokoyama A, Inoue H, Minami H, et al. Novel narrow-band imaging magnifying endoscopic classification for early gastric cancer. Dig Liver Dis. 2010;42:704–8.
61. Kobayashi M, Takeuchi M, Ajioka Y, Hashimoto S, Sato A, Narisawa R, Aoyagi Y. Mucin phenotype and narrow-band imaging with magnifying endoscopy for differentiated-type mucosal gastric cancer. J Gastroenterol. 2011;46(9):1064–70.
62. Kanemitsu T, Yao K, Nagahama T, Fujiwara S, Takaki Y, Ono Y, Matsushima Y, Matsui T, Tanabe H, Ota A, Iwashita A. The vessels within epithelial circle (VEC) pattern as visualized by magnifying endoscopy with narrow-band imaging (ME-NBI) is a useful marker for the diagnosis of papillary adenocarcinoma: a case-controlled study. Gastric Cancer. 2014;17:469–77.

63. Kanesaka T, Sekikawa A, Tsumura T, Maruo T, Osaki Y, Wakasa T, Shintaku M, Yao K. Absent microsurface pattern is characteristic of early gastric cancer of undifferentiated type: magnifying endoscopy with narrow-band imaging. Gastrointest Endosc. 2014;80:1194–8.
64. Okada K, Fujisaki J, Kasuga A, Omae M, Hirasawa T, Ishiyama A, Inamori M, Chino A, Yamamoto Y, Tsuchida T, Nakajima A, Hoshino E, Igarashi M. Diagnosis of undifferentiated type early gastric cancers by magnification endoscopy with narrow-band imaging. J Gastroenterol Hepatol. 2011;26:1262–9.
65. Yoshinaga S, Gotoda T, Oda I, Saito Y, Matsuda T, Nakajima T, et al. Clinical imaging of early gastric cancers—conventional endoscopy: including chromoendoscopy using indigo carmine. Stomach Intestine. 2009;44:650–62.
66. Nagahama T, Yao K, Maki S, et al. Usefull of magnifying endoscopy with narrow-band imaging for determining the horizontal extent of early gastric cancer when there is an unclear margin by chromoendoscopy. Gastrointest Endosc. 2011;75:1259–67.
67. Abe S, Oda I, Shimazu T, et al. Depth-predicting score for differentiated early gastric cancer. Gastric Cancer. 2011;14:35–40.
68. Choi J, Kim SG, Im JP, et al. Endoscopic prediction of tumor invasion depth in early gastric cancer. Gastrointest Endosc. 2011;73:917–27.
69. Choi J, Kim SG, Im JP, Kim JS, Jung HC, Song IS. Comparison of endoscopic ultrasonography and conventional endoscopy for prediction of depth of tumor invasion in early gastric cancer. Endoscopy. 2010;42:705–13.
70. Mocellin S, Marchet A, Nitti D. EUS for the staging of gastric cancer: a meta-analysis. Gastrointest Endosc. 2011;73:1122–34.
71. Yao K, Tominaga K, Doyama H. Current clinical feasibility of magnifying endoscopy for predicting early gastric cancer invasion depth. Stomach Intestine. 2015;50:616–8. in Japanese.

Chapter 15
Endoscopic Submucosal Dissection for Early Gastric Cancer: Getting It Right!

Ichiro Oda, Harushisa Suzuki, and Shigetaka Yoshinaga

Introduction

Endoscopic resection is widely accepted as an effective minimally invasive treatment technique for early gastric cancer (EGC) with a negligible risk of lymph node metastasis [1, 2]. Endoscopic resection preserves the functional stomach and therefore improves patient quality of life as compared to radical surgery. Remarkable progress has been made in the past decade in the field of endoscopic resection for gastric cancer, both in terms of expansion of the indications and in terms of improvements of the technique. The indications for endoscopic resection of EGC have been expanded based on an estimation of the risk of lymph node metastasis in cases of EGC from a large number of surgical cases [3, 4]. The technique of endoscopic resection has been improved from endoscopic mucosal resection (EMR) to endoscopic submucosal dissection (ESD) [5–11]. EMR procedures include inject and cut, strip biopsy, EMR with a cap-fitted endoscope, endoscopic aspiration mucosectomy and EMR with a ligating device, etc., while ESD is a relatively new endoscopic resection method that facilitates en bloc resection. This chapter addresses the indications, results, some technical tips, and complications of ESD for EGC.

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Indications of ESD for EGC

Endoscopic resection is generally indicated for EGC patients with a negligible risk of lymph node metastasis, because it is a local resection without lymph node dissection. The estimated incidence of lymph node metastasis in mucosal and submucosal gastric cancer is approximately 3 and 20 %, respectively [3, 4]. If patients who have zero risk of lymph node metastases could be identified with certainty, endoscopic resection would be the ideal treatment method for these patients. However, it is not possible to exclude the presence of lymph node metastasis by computed tomography (CT), positron emission tomography (PET), or endoscopic ultrasonography (EUS) with certainty, because lymph node metastases from EGC are often too small. As a result, the indication for endoscopic resection in patients with EGC is determined based on evaluation of clinicopathological findings that are considered to be associated with a negligible risk of lymph node metastasis. In the past, the accepted indications for endoscopic resection in cases of EGC were small intramucosal cancers measuring ≤2 cm in diameter, well to moderately differentiated histopathological type, and absence of ulceration [12]. In an effort to expand the indications, Gotoda et al. and Hirasawa et al. reviewed surgical cases and identified other characteristics of EGC that were associated with a negligible risk of lymph node metastasis, as follows [3, 4]:

- Well to moderately differentiated histopathological type, intramucosal cancer, >2 cm in diameter, without ulceration.
- Well to moderately differentiated histopathological type, intramucosal cancer, ≤3 cm in diameter, with ulceration.
- Poorly differentiated histopathological type, intramucosal cancer, ≤2 cm in diameter, without ulceration.
- Well to moderately differentiated histopathological type, ≤3 cm in diameter, minute submucosal (SM1) cancer.

Note that lymphovascular infiltration (ly(−), v(−)) should always be excluded as this finding correlates with a greatly increased risk of lymph node metastases. The histopathological type is classified as either well to moderately differentiated type (G1-G2) or poorly differentiated type (G3). The former includes papillary adenocarcinoma and tubular adenocarcinoma; the latter includes poorly differentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous adenocarcinoma.

Thus, these expanded indications now include larger lesions and lesions with ulceration. Such lesions were previously resected surgically because of the difficulty in effectively using EMR techniques for resection in this context. As a result, ESD was developed to achieve en bloc resection even for larger and ulcerative lesions.

Results

ESD is associated with a very high rate of en bloc resections, regardless of the tumor location, tumor size, and the presence of ulceration. In our previously published case series, we achieved a very high rate (98 %; 1008/1033 patients) of en bloc

resections [10]. Similarly, other studies have also reported high rates of en bloc resection, ranging from 92.7 to 98.0 % [11–21]. In addition, a meta-analysis revealed a higher rate of en bloc resection in patients undergoing ESD compared to those undergoing EMR [22]. After ESD, the curability is assessed based on the completeness of removal of the primary tumor and a zero possibility of lymph node metastasis. If the lesion was resected en bloc and is a ly(−), v(−) intramucosal cancer without ulceration, with negative resected margins, ≤2 cm in diameter, and shows well to moderately differentiated histopathological type, it can be considered a curative resection. If a lesion was resected en bloc with negative margins, is ly(−), v(−), and fulfills one of the following criteria:

1. >2 cm in diameter, well to moderately differentiated histopathological type, intramucosal, without ulceration, or
2. ≤3 cm in diameter, well to moderately differentiated histopathological type, intramucosal, with ulceration, or
3. ≤2 cm in diameter, poorly differentiated histopathological type, intramucosal, without ulceration, or
4. ≤3 cm in diameter, well to moderately differentiated histopathological type, SM1 invasion

then the resection can be considered an "extended-indication" curative resection. As for the long-term outcomes of curative resection and extended-indication curative resection, there have been few published reports with median follow-up periods of more than 5 years [23–27]. In our unit, we obtained very high 5-year disease-specific survival and 5-year overall survival rates in our case series [27].

Technical Tips Regarding ESD Performed for EGC

Basic Movement Is Dependent on ESD Device

Recently, a number of ESD devices have been developed that have just now become available for clinical use. These devices are divided into two major types: the IT-type knife and needle-type knife devices. The IT-type knife devices include the IT knife, IT knife 2, and IT knife nano (KD-611L, KD-611L, KD-612L; Olympus Medical Systems Corp., Tokyo, Japan) and the needle-type knife devices include the needle knife, hook knife, Dual Knife, (KD-1L-1, KD-620LR, KD-650L; Olympus Medical Systems Corp., Tokyo, Japan) and FlushKnife BT (DK2618JB; Fujifilm Corp., Tokyo, Japan), etc. Importantly, these two different types of devices require completely different approaches. The IT-type knife has an insulated ceramic ball at the tip of the knife, which prevents gastric wall perforation. Therefore, we use the metallic blade between the tip and the sheath while using the IT-type knife for cutting. When using the IT-type knife, it should be pulled from the far side to the near side before the metallic blade can be used for cutting purposes (Video 15.1). In contrast, ESD performed with a needle-type knife device requires an entirely different approach. Since the tip of the knife is not insulated, the tip can be used for cutting purposes.

Basically, the knife tip should be slid from the near side to the far side to avoid perforation (Video 15.2). Pulling of a needle-type knife from the far side to the near side, as in the case of the IT-type knife, is associated with a high risk of perforation.

These differences between the IT-type knife and needle-type knife are similar to the differences between a surgical knife and an electrosurgical knife. When a surgeon uses a surgical knife, it should be pulled from the far side to the near side for cutting purposes (Video 15.3); this corresponds to the use of the IT-type knife. In contrast, use of an electrosurgical knife corresponds to the use of a needle-type knife (Video 15.4).

ESD Strategy

The ESD procedure consists of the following steps (Figs. 15.1a–f and 15.2a–f):

1. The lesion margin is identified and marked at a distance of about 3–5 mm outside the margin by argon plasma coagulation or with a needle-type knife.
2. The mucosa is lifted by submucosal fluid injection followed by mucosal incision with an IT-type knife or needle-type knife.
3. Submucosal dissection under the lesion is performed with the IT-type knife or needle-type knife.

The setup of high-frequency electrical surgical units (ICC200, VIO330D; ERBE Corp., Tubingen, Germany), (ESG-100; Olympus Medical Systems Corp., Tokyo, Japan) depends on the endoscopist's preference. The setup used in our endoscopy unit is shown in Table 15.1.

When we use the IT-type knife as the main device for ESD, an initial incision is made at the far point (i.e., as shown on the TV monitor) with the needle-type knife, because the subsequent mucosal incision using the IT-type knife is then performed from the far side to the near side. When a lesion is located on the antrum, ESD is performed with a straight view, so the initial incision is made at the distal side of the lesion, which corresponds to the far side on the TV monitor (Fig. 15.1b). In contrast, the procedure is reversed for lesions located on the gastric body: the initial incision is made on the proximal side of the lesion, which corresponds to the far side on the TV monitor, because we use the retroflex view for lesions located on the gastric body (Fig. 15.2c). The mucosal incision is started from the point of the initial incision and pulled toward the near side. The IT-type knife should be placed in a tangential position parallel to the mucosa, and not in a vertical position, so as to provide for adequate depth of the mucosal incision (Video 15.1). The final step is the actual submucosal dissection under the lesion (Video 15.5). Submucosal dissection using the IT-type knife can also be performed from the far side on the TV monitor to the near side, because it is easier to dissect lengthwise rather than widthwise using the IT-type knife. Therefore, we start the submucosal dissection lengthwise from the far side to the near side (Fig. 15.3a, b). We then proceed to make a depression at the near side, after which we dissect widthwise hooking the IT-type knife on the edge

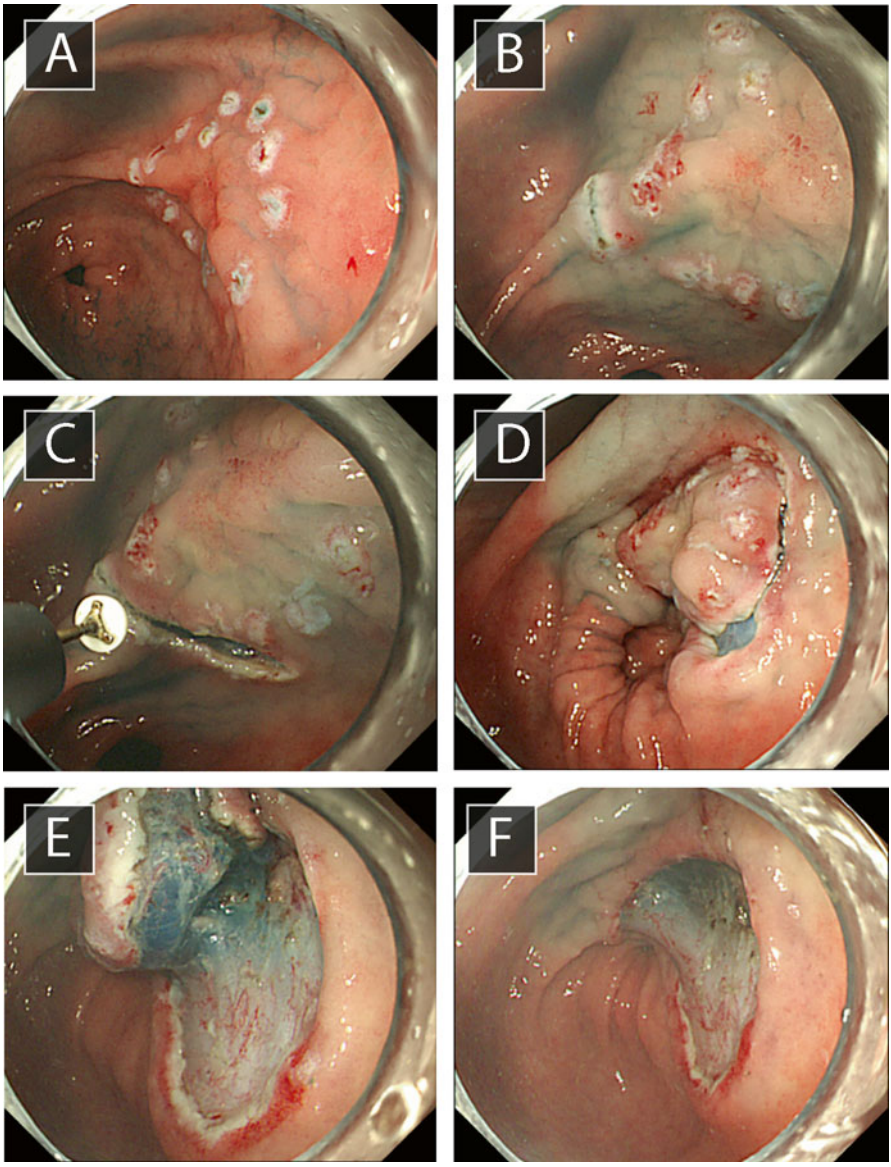

Fig. 15.1 Process of ESD with an IT-type knife for a lesion located on the posterior wall of the gastric antrum. (**a**) Marking of the periphery of the lesion, 3–5 mm away from the lateral margin of the lesion. This is followed by injection of fluid into the submucosal layer (not shown). (**b**) Initial incision at the far point with a needle-type knife. (**c**, **d**) Mucosal incision started from the point of the initial incision and pulled toward the near side. (**e**) Submucosal dissection with an IT-type knife. (**f**) Mucosal defect after the ESD

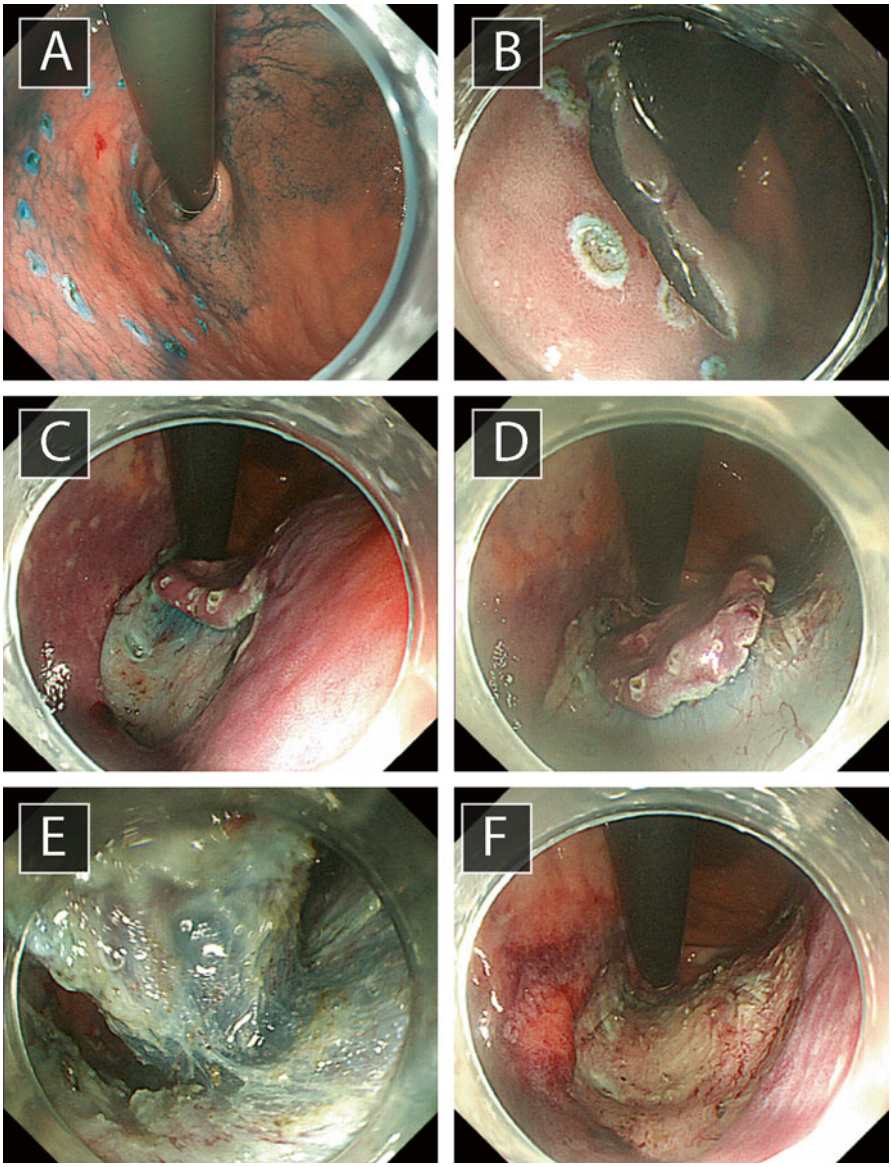

Fig. 15.2 Technique of ESD using an IT-type knife for a lesion located on the lesser curvature of the upper gastric body. (**a**) Marking of the periphery of the lesion 3–5 mm away from the lateral margin of the lesion. (**b**) After injection of fluid into the submucosal layer, the initial incision with the needle-type knife made at the far point. (**c**, **d**) Mucosal incision with the IT-type knife started from the point of the initial incision and pulled toward the near side. (**e**) Submucosal dissection performed with an IT-type knife. (**f**) Mucosal defect after the ESD

Table 15.1 Setup of high-frequency electric surgical unit of ESD for early gastric cancer

Procedure	Device	Mode	Output
(a) ICC200			
Marking	Needle-type knife	Forced coag	20 W
	APC	APC	1.8 L 40 W
Initial incision	Needle-type knife	ENDO CUT	Effect 3, 80 W
Mucosal incision	IT-type knife	ENDO CUT	Effect 3, 80 W
	Needle-type knife		
Submucosal dissection	IT-type knife	ENDO CUT	Effect 3, 80 W
		Forced coag	50 W
	Needle-type knife	ENDO CUT	Effect 3, 80 W
		Forced coag	50 W
Endoscopic hemostasis	IT-type knife	Forced coag	50 W
	Needle-type knife		
	Coagrasper	Soft coag	80 W
	Hot biopsy		
(b) VIO300D			
Marking	Needle-type knife	Swift coag	Effect 2, 50 W
	APC	Forced APC	1.8 L 40 W
Initial incision	Needle-type knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
Mucosal incision	IT-type knife	ENDO CUT I or Q	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50 W
	Needle-type knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50 W
Submucosal dissection	IT-type knife	ENDO CUT I or Q	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50 W
		Swift coag	Effect 5, 50 W
	Needle-type knife	ENDO CUT I	Effect 2, CUT duration 2, CUT interval 3
		DRY CUT	Effect 4, 50 W
		Swift coag	Effect 5, 50 W
Endoscopic hemostasis	IT-type knife	Swift coag	Effect 5, 50 W
	Needle-type knife		
	Coagrasper	Soft coag	Effect 5, 80 W
	Hot biopsy		
(c) ESG100			
Marking	Needle-type knife	Forced coag 1	20 W
Initial incision	Needle-type knife	Pulse cut slow	40 W
Mucosal incision	IT-type knife	Pulse cut slow	40 W
	Needle-type knife		

(continued)

Table 15.1 (continued)

Procedure	Device	Mode	Output
Submucosal dissection	IT-type knife	Pulse cut slow	40 W
		Forced coag 2	50 W
	Needle-type knife	Pulse cut slow	40 W
		Forced coag 2	50 W
Endoscopic hemostasis	IT-type knife	Forced coag 2	50 W
	Needle-type knife		
	Coagrasper	Soft coag	80 W
	Hot biopsy		

of the depression (Fig. 15.3c, d). It is important to always move parallel to the gastric wall curvature to avoid perforation.

In contrast, the procedure is reversed when the needle-type knife is used as the main device for ESD. The incision is started from the near side on the TV monitor and then the knife tip is slid from the near side to the far side (Video 15.2). Submucosal dissection using needle-type knife is also performed from the near side to the far side (Video 15.6). Thus, ESD requires a completely different approach, depending on which type of device is being used during the procedure.

Training for ESD

ESD requires a high level of technical expertise and it may appear technically challenging to less experienced endoscopists. ESD trainees at the National Cancer Center Hospital in Tokyo, Japan, follow a step-by-step process for learning ESD techniques. The first step entails acquiring a basic knowledge and understanding of EGC and ESD, in particular, the diagnosis of EGC and the indications for ESD. The next step is for trainees to observe expert endoscopists in action as they perform various ESD procedures. The third step involves trainees acquiring first-hand experience by assisting experts during actual ESDs. This is followed by the fourth step, in which ESD training is further refined using animal models. In the final step, it is important for trainees to start performing ESDs on lesions that technically less challenging, including those that are located in the lower third of the stomach, those that are small in size and do not show ulcer fibrosis. Trainees perform their first 10 ESDs with direct hands-on support from highly qualified endoscopists, and then start to perform ESDs by themselves with mostly verbal guidance from expert endoscopists. As their ESD technique improves, trainees are gradually assigned to perform ESDs on lesions located in the middle and upper thirds of the stomach and lesions that are larger in size. Based on our training program, the step-by-step training system at our center has been highly effective, with an *en bloc* resection rate of 100 % and a low complication rate. As a result of this program, the point on the learning curve at which our trainees acquire the basic technical skills for successfully performing ESD in the lower third of the stomach was 30 cases [28].

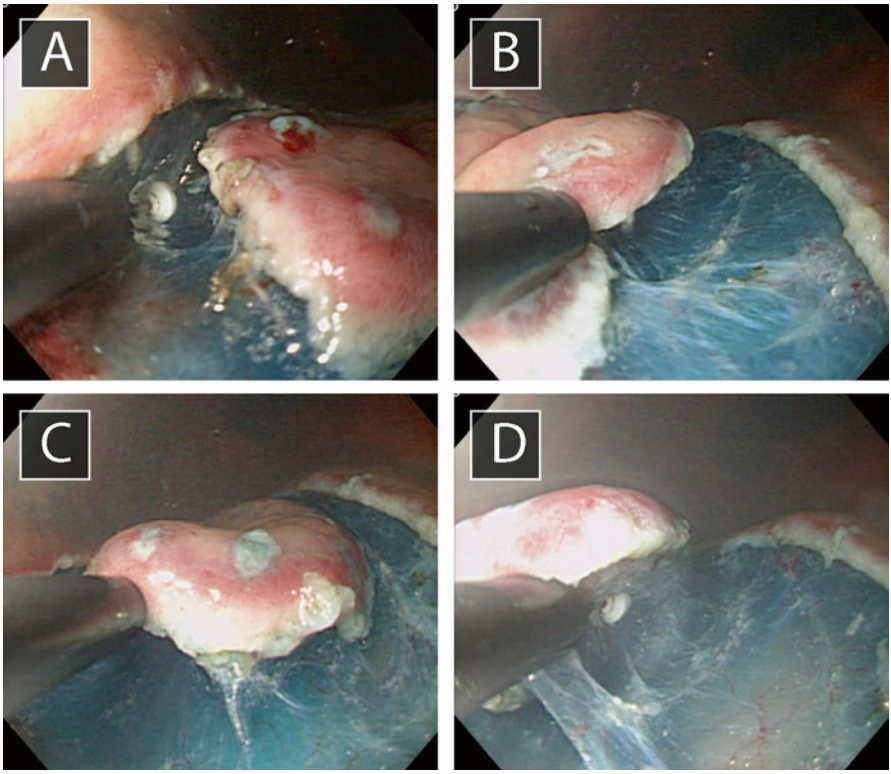

Fig. 15.3 Detailed procedure of submucosal dissection with the IT-type knife. (**a**, **b**) Submucosal dissection with the IT-type knife started lengthwise from the far side to the near side, with the depression made at the near side. (**c**, **d**) Submucosal dissection performed widthwise by hooking the IT-type knife on the edge of the depression

Complications

ESD is associated with a relatively high risk of complications. Bleeding and perforation are the two major complications. Endoscopists must be aware of not only the risk factors for and the incidence of complications, but also know how to effectively manage complications.

Perforation

Most perforations occur during ESD and the reported risk of perforation is in the range of 1.2–5.2 % for gastric ESD [10, 15, 16, 19–21, 29–34]. In terms of delayed perforation occurring after the completion of gastric ESD, in one study, such perforations occurred in six (0.5 %) of 1159 consecutive patients with 1329 EGCs treated by ESD [35]. The possible mechanisms for perforations induced by ESD are

unanticipated injury of the muscularis propria caused by insufficient submucosal injection or miscalculation of the gastric wall curvature. In order to avoid perforation, adequate space in the submucosal layer between the muscularis propria and the mucosal layer is essential. To this end, a sufficient amount of submucosal injection solution must be injected. In order to lift the mucosa for the longer procedure period required by ESD, the effectiveness of sodium hyaluronate, glycerol, or a combination of sodium hyaluronate and glycerol has been reported previously [11, 36–38]. The use of an injection solution mixed with indigocarmine dye for submucosal injection is effective for better recognition of the gastric wall curve because it allows distinction of the (white) muscularis propria from the (blue) submucosal layer. The use of a transparent attachment to the scope to lift the mucosal layer is also useful for recognizing the gastric wall curve. If perforation is recognized during the procedure, endoscopic clipping can be performed (Fig. 15.4a, b). In the past, gastric perforations occurring during endoscopic resections of early cancers invariably necessitated emergency surgery, which effectively meant that all the benefits of endoscopic resection were lost. Endoscopic clips were originally developed for hemostatic purposes [39]. Closure of a perforation using such clips after snare excision of a gastric leiomyoma was first reported by Binmoeller et al. in 1993 [40]. In 2006, endoscopic closure with endoscopic clips for endoscopic resection-related gastric perforations was reported to be effective in a series of consecutive cases [29]. In that study, 115 (98.3 %) of 117 patients with gastric perforations were successfully treated conservatively by the use of endoscopic clips for closure of the perforations.

Bleeding

Bleeding complications associated with ESD can be subdivided into immediate (intraoperative) bleeding occurring during the procedure and delayed bleeding occurring after the procedure. Immediate bleeding is infrequent with EMR but is quite common with ESD. Management of immediate bleeding plays a critical role in the successful completion of ESD. Electrocautery is used for hemostasis in cases of immediate bleeding occurring during ESD, because endoscopic clips interfere with the subsequent resection procedure [41, 42]. Electrocautery is usually carried out using different devices depending on the degree of bleeding. Minor oozing can be controlled by electrocautery using a cutting device, such as the IT knife 2, Hook knife, Dual knife, FlushKnife BT (Video 15.7). It is also necessary to precoagulate to prevent bleeding using a cutting device when vessels are found during the procedure. Electrocautery using hemostatic forceps, such as the Coagrasper (FD-410LR; Olympus Medical Systems Corp) or hot biopsy forceps (Radial Jaw; Boston Scientific Japan Corp, Tokyo, Japan), is suitable for arterial bleeding (Video 15.7). The critical step for achieving adequate hemostasis is identification of the exact bleeding point using water flushing. Endoscopes equipped with water-jet systems (GIF-Q260J; Olympus Medical Systems Corp.; EG-450RD5; Fujifilm Corp., Tokyo, Japan), which are useful for precisely determining the bleeding point have recently become available for clinical use.

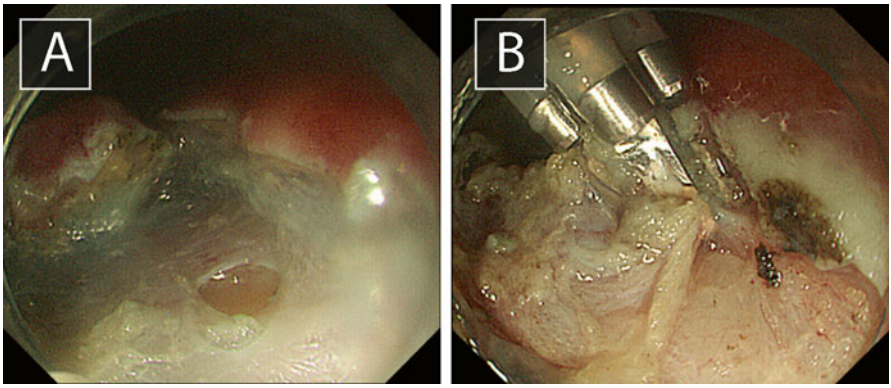

Fig. 15.4 Endoscopic closure of a gastric wall perforation that occurred during ESD. (**a**) A small perforation measuring 3 mm in size that occurred during gastric ESD. (**b**) Perforation closed successfully with endoscopic clips

The reported incidence of delayed bleeding after ESD is in the range of 0–15.6 % [10, 15, 16, 19–21, 30–34, 43, 44]. This wide range is partly due to differences in the definition of delayed bleeding among reported studies. If delayed bleeding were defined as bleeding requiring endoscopic treatment with any clinical symptoms of bleeding such as hematemesis and melena, the reported incidence is 3–8 % [10, 20, 21, 30, 32–34, 43]. All endoscopic treatment modalities can also be used individually or in combination for achieving hemostasis in cases of delayed bleeding after endoscopic resection. Different modalities are applied according to the time of onset of delayed bleeding. In cases with delayed bleeding occurring in the early phase after ESD, the artificial ulcer floor is still soft with little granulation tissue, so that endoscopic clips or electrocautery using hemostatic forceps can be applied to control the bleeding. In cases of delayed bleeding occurring in the later phase after ESD, the artificial ulcer is floor is hard with granulation tissue, so the injection method is preferably used to control bleeding.

Conclusions

Endoscopic resection is indicated for EGCs with a negligible risk of lymph node metastasis. ESD offers the advantage of achieving en bloc resection. Step-by-step training is important for training in ESD techniques. The technique employed for ESD depends on which type of device (IT-type knife or needle-type knife) is used during the procedure. ESD is associated with a relatively high risk of complications. Endoscopists must be aware of not only the incidence and risk factors for complications, but they must also know how to effectively manage these complications.

References

1. Soetikno R, Kaltenbach T, Yeh R, et al. Endoscopic mucosal resection for early cancers of the upper gastrointestinal tract. J Clin Oncol. 2005;23:4490–8.
2. Gotoda T, Ho KY, Soetikno R, et al. Gastric ESD: current status and future directions of devices and training. Gastrointest Endosc Clin N Am. 2014;24:213–33.
3. Gotoda T, Yanagisawa A, Sasako M, et al. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. Gastric Cancer. 2000;3:219–25.
4. Hirasawa T, Gotoda T, Miyata S, et al. Incidence of lymph node metastasis and the feasibility of endoscopic resection for undifferentiated-type early gastric cancer. Gastric Cancer. 2009;12:148–52.
5. Tada M, Murakami A, Karita M, et al. Endoscopic resection of early gastric cancer. Endoscopy. 1993;25:445–51.
6. Inoue H, Takeshita K, Hori H, et al. Endoscopic mucosal resection with a cap-fitted panendoscope for esophagus, stomach, and colon mucosal lesions. Gastrointest Endosc. 1993;39:58–62.
7. Tanabe S, Koizumi W, Kokutou M, et al. Usefulness of endoscopic aspiration mucosectomy as compared with strip biopsy for the treatment of gastric mucosal cancer. Gastrointest Endosc. 1999;50:819–22.
8. Suzuki H. Endoscopic mucosal resection using ligating device for early gastric cancer. Gastrointest Endosc Clin N Am. 2001;11:511–8.
9. Ono H, Kondo H, Gotoda T, et al. Endoscopic mucosal resection for treatment of early gastric cancer. Gut. 2001;48:225–9.
10. Oda I, Gotoda T, Hamanaka H, et al. Endoscopic submucosal dissection for early gastric cancer: technical feasibility, operation time and complications from a large consecutive series. Dig Endosc. 2005;17:54–8.
11. Yamamoto H, Kawata H, Sunada K, et al. Successful one-piece resection of large superficial tumors in the stomach and colon using sodium hyaluronate and small-caliber-tip transparent hood. Endoscopy. 2003;35:690–4.
12. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). Gastric Cancer. 2011;14:113–23.
13. Oka S, Tanaka S, Kaneko I, et al. Advantage of endoscopic submucosal dissection compared with EMR for early gastric cancer. Gastrointest Endosc. 2006;64:877–83.
14. Imagawa A, Okada H, Kawahara Y, et al. Endoscopic submucosal dissection for early gastric cancer: results and degrees of technical difficulty as well as success. Endoscopy. 2006;38:987–90.
15. Oda I, Saito D, Tada M, et al. A multicenter retrospective study of endoscopic resection for early gastric cancer. Gastric Cancer. 2006;9:262–70.
16. Isomoto H, Shikuwa S, Yamaguchi N, et al. Endoscopic submucosal dissection for early gastric cancer: a large-scale feasibility study. Gut. 2009;58:331–6.
17. Goto O, Fujishiro M, Kodashima S, et al. Outcomes of endoscopic submucosal dissection for early gastric cancer with special reference to validation for curability criteria. Endoscopy. 2009;41:118–22.
18. Nakamoto S, Sakai Y, Kasanuki J, et al. Indications for the use of endoscopic mucosal resection for early gastric cancer in Japan: a comparative study with endoscopic submucosal dissection. Endoscopy. 2009;41:746–50.
19. Chung IK, Lee JH, Lee SH, et al. Therapeutic outcomes in 1000 cases of endoscopic submucosal dissection for early gastric neoplasms: Korean ESD Study Group multicenter study. Gastrointest Endosc. 2009;69:1228–35.
20. Akasaka T, Nishida T, Tsutsui S, et al. Short-term outcomes of endoscopic submucosal dissection (ESD) for early gastric neoplasm: multicenter survey by osaka university ESD study group. Dig Endosc. 2011;23:73–7.

21. Ahn JY, Jung HY, Choi KD, et al. Endoscopic and oncologic outcomes after endoscopic resection for early gastric cancer: 1370 cases of absolute and extended indications. Gastrointest Endosc. 2011;74:485–93.
22. Park YM, Cho E, Kang HY, et al. The effectiveness and safety of endoscopic submucosal dissection compared with endoscopic mucosal resection for early gastric cancer: a systematic review and metaanalysis. Surg Endosc. 2011;25:2666–77.
23. Oda I, Oyama T, Abe S, et al. Preliminary results of multicenter questionnaire study on long-term outcomes of curative endoscopic submucosal dissection for early gastric cancer. Dig Endosc. 2014;26:214–9.
24. Kosaka T, Endo M, Toya Y, et al. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a single-center retrospective study. Dig Endosc. 2014;26:183–91.
25. Tanabe S, Ishido K, Higuchi K, et al. Long-term outcomes of endoscopic submucosal dissection for early gastric cancer: a retrospective comparison with conventional endoscopic resection in a single center. Gastric Cancer. 2014;17:130–6.
26. Ohnita K, Isomoto H, Shikuwa S, et al. Early and long-term outcomes of endoscopic submucosal dissection for early gastric cancer in a large patient series. Exp Ther Med. 2014;7:594–8.
27. Suzuki H, Oda I, Abe S et al (2015) High rate of 5-year survival among patients with early gastric cancer undergoing curative endoscopic submucosal dissection. Gastric Cancer. [Epub ahead of print].
28. Oda I, Odagaki T, Suzuki H, et al. Learning curve for endoscopic submucosal dissection of early gastric cancer based on trainee experience. Dig Endosc. 2012;24 Suppl 1:129–32.
29. Minami S, Gotoda T, Ono H, et al. Complete endoscopic closure of gastric perforation induced by endoscopic resection of early gastric cancer using endoclips can prevent surgery. Gastrointest Endosc. 2006;63:596–601.
30. Jung HY, Choi KD, Song HJ, et al. Risk management in endoscopic submucosal dissection using needle knife in Korea. Dig Endosc. 2007;19 Suppl 1:S5–8.
31. Takenaka R, Kawahara Y, Okada H, et al. Risk factors associated with local recurrence of early gastric cancers after endoscopic submucosal dissection. Gastrointest Endosc. 2008;68: 887–94.
32. Ono H, Hasuike N, Inui T, et al. Usefulness of a novel electrosurgical knife, the insulation-tipped diathermic knife-2, for endoscopic submucosal dissection of early gastric cancer. Gastric Cancer. 2008;11:47–52.
33. Sugimoto T, Okamoto M, Mitsuno Y, et al. Endoscopic submucosal dissection is an effective and safe therapy for early gastric neoplasms: a multicenter feasible study. J Clin Gastroenterol. 2012;46:124–9.
34. Toyokawa T, Inaba T, Omote S, et al. Risk factors for perforation and delayed bleeding associated with endoscopic submucosal dissection for early gastric neoplasms; analysis of 1123 lesions. J Gastroenterol Hepatol. 2012;27:907–12.
35. Hanaoka N, Uedo N, Ishihara R, et al. Clinical features and outcomes of delayed perforation after endoscopic submucosal dissection for early gastric cancer. Endoscopy. 2010;42: 1112–5.
36. Hyun JJ, Chun HR, Chun HJ, et al. Comparison of the characteristics of submucosal injection solutions used in endoscopic mucosal resection. Scand J Gastroenterol. 2006; 41:488–92.
37. Fujishiro M, Yahagi N, Kashimura K, et al. Comparison of various submucosal injection solutions for maintaining mucosal elevation during endoscopic mucosal resection. Endoscopy. 2004;36:579–83.
38. Fujishiro M, Yahagi N, Kashimura K, et al. Tissue damage of different submucosal injection solutions for EMR. Gastrointest Endosc. 2005;62:933–42.
39. Hachisu T. Evaluation of endoscopic hemostasis using an improved clipping apparatus. Surg Endosc. 1988;2:13–7.

40. Binmoellar KF, Grimm H, Soehendra N. Endoscopic closure of a perforation using metallic clips after snare excision of gastric leiomyoma. Gastrointest Endosc. 1993;39:172–4.
41. Toyonaga T, Nishino E, Hirooka T, et al. Intraoperative bleeding in endoscopic submucosal dissection in the stomach and strategy for prevention and treatment. Dig Endosc. 2006;18:S123–7.
42. Oda I, Suzuki H, Nonaka S, et al. Complications of gastric endoscopic submucosal dissection. Dig Endosc. 2013;25 Suppl 1:71–8.
43. Takizawa K, Oda I, Gotoda T, et al. Routine coagulation of visible vessels may prevent delayed bleeding after endoscopic submucosal dissection—an analysis of risk factors. Endoscopy. 2008;40:179–83.
44. Hotta K, Oyama T, Akamatsu T, et al. A comparison of outcomes of endoscopic submucosal dissection (ESD) for early gastric neoplasms between high-volume and low-volume centers: multi-center retrospective questionnaire study conducted by the Nagano ESD Study Group. Intern Med. 2010;49:253–9.

Chapter 16
The Japanese Viewpoint on the Histopathology of Early Gastric Cancer

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Introduction

The incidence of gastric cancer is significantly higher in Eastern Asian countries than it is in Western countries [1]. The high infection rate of *Helicobacter pylori (H. pylori*) is thought to be a major cause of the high incidence of gastric cancer in Japan [2, 3]. These days though, following the decreasing *H. pylori* infection rate, the age-standardized incidence and mortality rates have been continuously declining [4, 5]. However, the total number of gastric cancer patients still shows a slightly increasing trend mainly because of higher life expectancy. For this reason, gastric cancer remains the second leading cause of cancer death in Japan [5].

Early gastric cancer is defined as any neoplastic lesion restricted to the gastric mucosa or submucosa, irrespective of the presence of lymph node metastasis [6, 7]. Early gastric cancer demonstrates a significantly improved outcome compared to more advanced lesions, because these early lesions are largely curable by local resection [8]. Introduction of a number of diagnostic methods, including double contrast radiography as well as conventional and chromoendoscopy, has significantly

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improved the sensitivity of early stage gastric cancer detection [9, 10]. Currently, early gastric cancer accounts for more than half of all gastric cancers diagnosed in Japan. This proportion is markedly higher than in Western countries [5].

Histological Diagnosis of Adenoma and Intramucosal Carcinoma in Japan

It is well recognized that Japanese and European/American pathologists use different criteria to diagnose intramucosal adenocarcinoma [11–13]. First, there is a difference in the classification of gastric intramucosal neoplasms between the Japanese Classification of Gastric Carcinoma (JCGC) and the World Health Organization (WHO) classification [6, 11, 14]. The WHO classification uses the terms "dysplasia" and "adenoma" to represent nonpolypoid and polypoid noninvasive neoplasms, respectively. However, in the JCGC, adenoma is the only term used for benign glandular neoplasms, regardless of their gross morphology and the term is applied to a relatively limited range of lesions, as detailed later. According to JCSC criteria, all noninvasive glandular neoplasms that do not fulfill the Japanese definition of adenoma are therefore classified by default as adenocarcinoma. As a result, intramucosal gastric cancer in Japan comprises a wider range of lesions than would be included in this category in the West. This includes, for example, all cases of high-grade dysplasia and even a subset of low-grade dysplasias and adenomas as diagnosed by Western pathologists.

Where does this difference derive from? In Western countries, the presence of stromal invasion is absolutely required for a diagnosis of adenocarcinoma. By contrast, Japanese pathologists establish a diagnosis of adenocarcinoma based on cytological and structural atypia, irrespective of the presence or absence of invasion. In spite of this difference, the exact cutoff between high-grade dysplasia and intramucosal adenocarcinoma may not be a major issue in the routine clinical setting of a biopsy-based diagnosis, since both high-grade dysplasia and intramucosal adenocarcinoma mandate endoscopic resection. Furthermore, a recent study demonstrated that lesions showing histological features characteristic of high-grade dysplasia in the mucosal layer are associated with risks of submucosal invasion and lymphovascular permeation, similar to intramucosal adenocarcinoma as defined by Western criteria [15]. From a pragmatic viewpoint, this supports the practice of lumping these two categories.

If the presence of invasion is not required for a diagnosis of adenocarcinoma, where and how do Japanese pathologists draw the line between adenoma and adenocarcinoma? The basic concept is that if a mucosal lesion shows cytological and/or architectural abnormalities that could also be seen in adenocarcinoma invading the submucosal layer, the lesion must be viewed and treated as an adenocarcinoma. This concept stems from experience with bona fide submucosal gastric cancers, which lack evidence of invasion in the overlying mucosal component of the lesion. In other words, a mucosal lesion may not necessarily express morphologic features

indicating invasion, but may nonetheless unequivocally demonstrate the biologic capacity to invade and metastasize. To mandate resection of lesions with a risk of invasion, the term carcinoma is preferred even if evidence of invasion is absent in biopsy specimens. In short, the difference in diagnostic criteria for adenocarcinoma of the upper gastrointestinal tract between Japan and Western countries derives from the fact that Japanese pathologists place emphasis on different morphologic features to predict poor clinical outcome rather than identification of the exact evidence of stromal invasion.

In Japan, the diagnostic term adenoma is applied to a limited range of lesions. The classical morphology of intestinal-type adenoma recognized by Japanese pathologists is fundamentally characterized by cytological features of complete-type intestinal metaplasia and low-grade cytological atypia (Fig. 16.1a) [14]. These lesions typically have uniform, basally oriented nuclei and superficially localized proliferative activity, and they are often associated with cystically dilated glands in the deeper mucosal layer. If a lesion of this kind shows high-grade cytological atypia, these are regarded as intramucosal adenocarcinoma rather than high-grade adenoma. Adenomas diagnosed based on these criteria are generally thought to be associated with a minimal risk of invasion or progression to invasive adenocarcinoma and may therefore be followed up without resection [16, 17]. However, a recent study reported that a subset of lesions pathologically diagnosed as adenoma using Japanese criteria may display areas of high-grade intramucosal neoplasia, particularly if the lesion was large (>20 mm), or if it showed redness or a depressed area endoscopically [18]. Although all of these lesions were shown to be noninvasive after examination of endoscopically removed specimens, this does suggest that a subset of intestinal-type adenoma may show more aggressive features.

Intestinal-type adenoma had long been the only histological subtype of gastric adenoma recognized by the JCGC. However, pyloric gland adenoma has now been added as a novel subtype of gastric adenoma to the most recent classification (Fig. 16.1b, c) [14]. Increasingly, pyloric gland adenomas are recognized as a clinicopathologically and genetically distinct subtype of gastric adenoma [19–21]. Because of the significant risk of progression to adenocarcinoma, endoscopic removal is recommended for all pyloric gland adenomas, unlike intestinal-type adenomas. Lastly, even though the WHO classification lists foveolar-type adenoma as a third adenoma subtype, this variant is not included in the JCGC. Regrettably, a detailed description of the JCGC adenoma classification is not included in the official English-language version.

Group Classification for Gastric Biopsy

In Japan, the five-tiered "Group Classification" is widely used in the histopathological examination of endoscopic biopsy specimens [6]. This classification was recently modified to remain consistent with the revised Vienna Classification [13, 22]. The updated Group Classification is shown as follows:

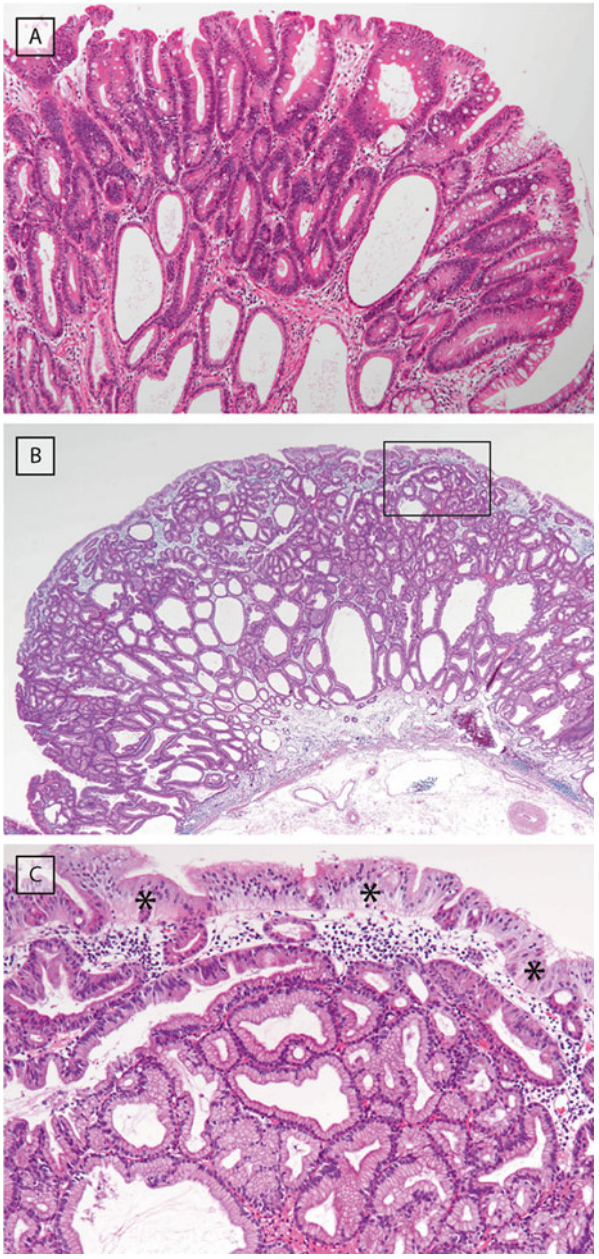

Fig. 16.1 Intestinal-type adenoma and pyloric gland adenoma. (**a**) Intestinal-type adenoma shows small intestinal differentiation and consists of absorptive and goblet cells. Nuclei are uniform and basally located. Cystically dilated glands are observed in the deeper layer. (**b**) Pyloric gland adenoma consisting of proliferation of closely packed pyloric glands (*box* shown in **c**). (**c**) The overlying epithelium of pyloric gland adenomas characteristically shows foveolar differentiation (*asterisks*)

Group X: Inappropriate material for which histological diagnosis cannot be made.
Group 1: Normal tissue or nonneoplastic lesion.
Group 2: Material for which diagnosis of neoplastic or nonneoplastic lesion is difficult. In such a case, the pathologist should describe the lesion as "indefinite for neoplasia" and add the following reasons for clinicians:

1. Atypical cells exist, but a diagnosis of neoplasia based on cellular atypia is difficult because of the small volume.
2. Atypical cells exist, but a diagnosis of neoplastic or nonneoplastic lesion is difficult due to remarkable erosion and/or inflammation.
3. Atypical cells exist, but a diagnosis of neoplastic or nonneoplastic lesion is difficult due to tissue damage.

Group 3: Adenoma.
Group 4: Neoplastic lesion that is suspected to be carcinoma.
Group 5: Carcinoma.

The updated Group Classification is directly related to clinical management. For instance, a diagnosis of Group 2 requires rebiopsy, sometimes followed by therapy for peptic ulcer disease. A diagnosis of Group 4 indicates a neoplastic lesion that cannot be classified as either adenoma or adenocarcinoma, and thus requires endoscopic resection or rebiopsy to obtain a definitive histologic diagnosis. Notably, a Group 5 diagnosis includes noninvasive lesions, as per the definition in the JCGC, and therefore does not fully correspond to Category 5 lesions of the Vienna Classification [6, 22].

Diagnosis of "Low-Grade Adenocarcinoma"

Japanese pathologists have a lower threshold for the diagnosis of gastric adenocarcinoma and will sometimes diagnose carcinoma on biopsy specimens that Western pathologists would sign out as low-grade dysplasia [12]. This is due in part to increased awareness of possible discordance between morphological abnormalities and biological aggressiveness in gastric neoplasia, as explained earlier. In most instances of colorectal adenocarcinoma, tumors exhibit significant cytological and architectural atypia, which together reflect malignant transformation. By contrast, in gastric cancer malignant behavior is often not reflected in high-grade cellular atypia and/or architectural disarray. Some gastric cancers are frankly invasive despite relatively bland cytological features. Clearly this can lead to underdiagnosis and treatment (Fig. 16.2a, b) [23]. Intratumoral heterogeneity is another potential diagnostic difficulty. It is quite common to observe an area of poorly differentiated adenocarcinoma situated deep to a differentiated-type intramucosal component. Notably, differentiated-type adenocarcinomas with a gastric epithelial phenotype are more likely to have poorly differentiated components and to show lymph node metastasis [24–26]. On account of this, Japanese pathologists are conscious of potential underdiagnosis of adenocarcinoma with a gastric foveolar phenotype.

A low-grade adenocarcinoma subtype showing intestinal epithelial differentiation is worthy of special consideration. A lesion that poses diagnostic difficulty in histological as well as endoscopic examination, this subtype is well known to Japanese pathologists and endoscopists and is described in the English medical literature as a "very well-differentiated gastric carcinoma of intestinal type"

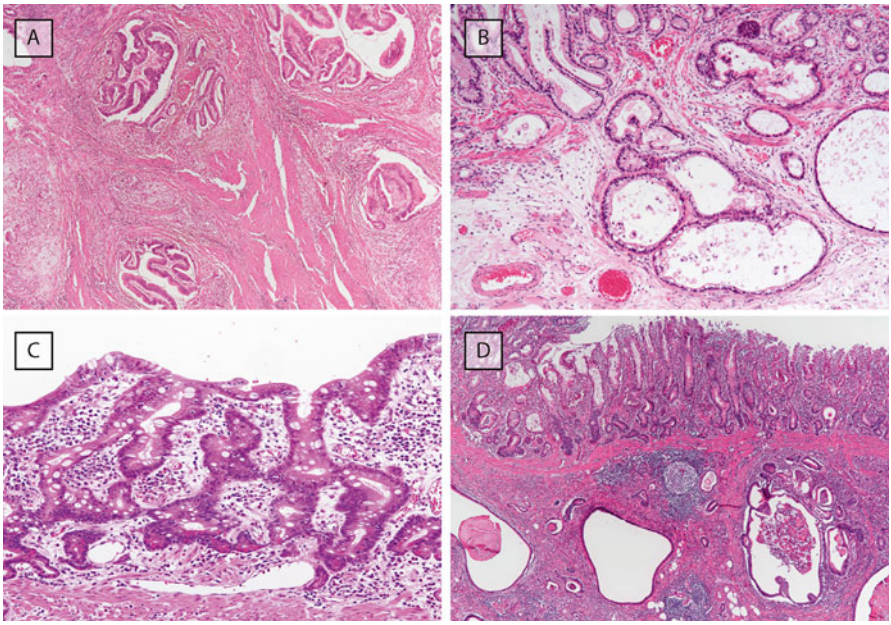

Fig. 16.2 Tubular adenocarcinoma with gastric foveolar epithelial differentiation and "very well-differentiated adenocarcinoma of intestinal type." (**a**) Tumor cells show features of foveolar epithelial cells and are arranged in a tubulo-papillary pattern. The nuclear/cytoplasmic ratio is low. Tumor glands are surrounded by prominent stromal fibrosis. (**b**) Another case of adenocarcinoma with foveolar epithelial differentiation invading the submucosal layer. Tumor cells show low nuclear/cytoplasmic ratio and have clear cytoplasm. (**c**) Case of a "very well-differentiated adenocarcinoma of intestinal type." The tumor cells show intestinal epithelial differentiation consisting of absorptive, goblet, and Paneth cells. Notice the irregular branching/fusion of neoplastic glands. Tumor cells show low nuclear/cytoplasmic ratio and minimal nuclear atypia. (**d**) A "very well-differentiated adenocarcinoma of intestinal type" case showing submucosal invasion. Irregularly shaped tumor glands proliferate in the submucosal layer in association with prominent fibrosis

(Fig. 16.2c, d) [27–29]. This type of adenocarcinoma occurs predominantly in the midgastric body, often on a background of intestinalized mucosa and endoscopically appears as a shallow depression. These lesions generally exhibit low-grade cytological atypia and morphological features of intestinalized epithelium, sometimes admixed with gastric foveolar-type cells. Because of the minimal cytological atypia, recognizing the characteristic architectural features is important. These include tortuous, branched, and/or anastomosing glands and the presence of microcystic distended glands. Very well-differentiated gastric carcinoma of intestinal type are generally indolent tumors and mostly limited to the mucosal layer. However, interestingly, Ushiku et al. showed that lesions with a mixed intestinal and gastric phenotype tended to display poorly differentiated components and show submucosal invasion [29].

Special Types of Gastric Carcinoma

Several rare but important subtypes of gastric carcinoma show distinct clinicopathological features, including adenosquamous/squamous cell carcinoma, neuroendocrine carcinoma, hepatoid adenocarcinoma, and choriocarcinoma. However, these are very aggressive neoplastic lesions and usually present clinically at advanced stages of disease.

An exception is carcinoma with lymphoid stroma, which is often diagnosed at earlier stages. Carcinoma with lymphoid stroma is characterized by dense lymphocytic infiltration, which is commonly associated with secondary lymphoid follicle formation (Fig. 16.3) [30]. Carcinoma with lymphoid stroma constitutes approximately 3 % of all gastric cancers in Japan and typically shows expansive growth and poorly or moderately differentiated histology. Intramucosal lesions often show a unique histological feature referred to as a "lace pattern" [31]. Endoscopic findings are also characteristic and often show submucosal tumor-like appearance reflecting its expansive growth. Carcinoma with lymphoid stroma is commonly associated with Epstein–Barr virus (EBV) infection, which can be demonstrated by in situ hybridization for EBER-1. Recent studies also revealed characteristic genetic

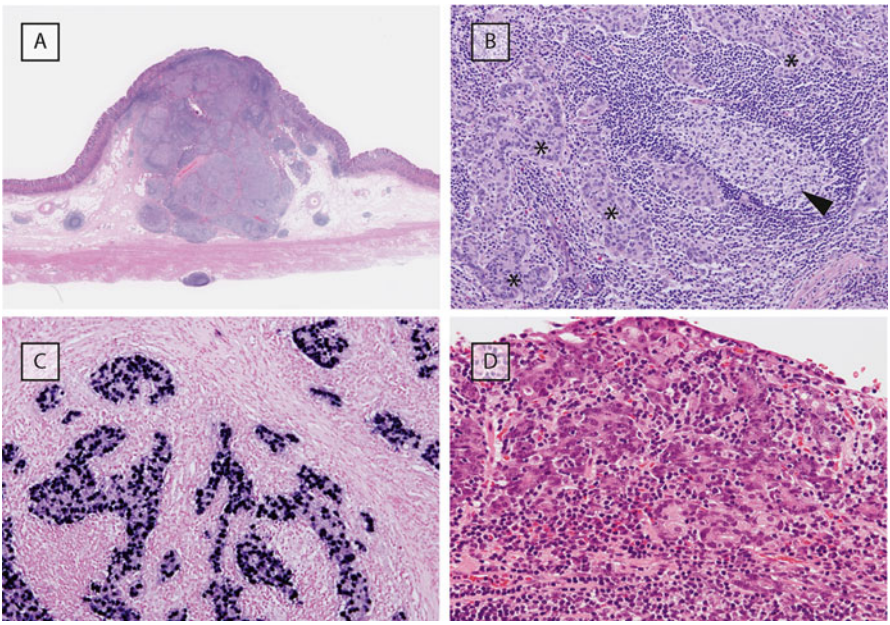

Fig. 16.3 Carcinoma with lymphoid stroma. (**a**) The tumor shows expansive growth in the submucosal layer. (**b**) Poorly differentiated tumor nests (*asterisks*) surrounded by dense lymphocytic infiltration and lymphoid follicle (*arrowhead*). (**c**) In situ hybridization for EBER-1 shows diffuse positivity (*dark blue*/*black* stain) in tumor cells. (**d**) Intramucosal EBV-positive adenocarcinoma forming anastomosing cords of cancer cells, referred to as the "lace pattern." Notice the prominent intraepithelial lymphocytes

features of EBV-positive gastric cancers, including frequent *PIK3CA* mutations [32]. EBV-associated gastric cancers have a better prognosis and lower risk for lymph node metastasis compared to conventional-type adenocarcinomas [33]. This may relate to the prominent lymphoid antitumor response.

Histological Evaluation of Endoscopically Resected Specimens

Endoscopic resection has become the reference standard in the treatment of superficial gastric cancers. Particularly, the introduction of endoscopic submucosal dissection (ESD) has enabled en bloc resection of larger lesions [34, 35]. As a result of this technical advance, endoscopic resection is now technically feasible for a wider range of gastric lesions. In turn the accurate assessment of curability on the endoscopic resection specimen becomes an evermore important issue.

The current Japanese gastric cancer treatment guidelines stipulate the following minimum dataset to assess the curability of endoscopic resection, all based on histopathological examination: (1) status of resection margin, (2) tumor size, (3) depth of invasion, (4) the presence of ulcer scar, (5) lymphovascular invasion, and (6) histological type [36]. These histopathological parameters have been derived from extensive analyses of surgically resected early gastric cancers. Particularly, a study conducted by Gotoda et al., involving 5265 cases of early gastric cancer made a major contribution to the development of the initial guideline [37]. This study analyzed possible correlations between clinicopathological parameters and the presence of lymph node metastases, and suggested features that are associated with a virtually negligible risk of lymph node metastasis. All endoscopically resected specimens are histologically examined to determine the minimum dataset outlined earlier. Current guidelines further discern criteria for "Curative resection" and criteria for "Curative resection for tumors of expanded indications" although lesions fulfilling either set of criteria are now generally treated by endoscopic resection (Fig. 16.4) [36].

In the assessment of curability, the importance of resection margins is self-evident. However, in case of a positive lateral margin in a small area, close follow-up can be an option [38]. The measurement of tumor size is a straightforward procedure, although accurate measurement of the maximum tumor diameter does require precise mapping of the lesion (Fig. 16.5a, b). In Japan, this meticulous exercise is done routinely. When a tumor involves the submucosal layer, the depth of submucosal invasion must be recorded. To facilitate objective and reproducible evaluation, we often use immunohistochemistry for desmin and measure the absolute distance between the lower end of the muscularis mucosae and the deepest portion of the tumor (Fig. 16.5c).

The presence of lymphovascular permeation is strongly associated with lymph node metastasis [37, 39, 40], but reliable evaluation of lymphatic and/or vascular permeation is often difficult on routine hematoxylin and eosin-stained sections.

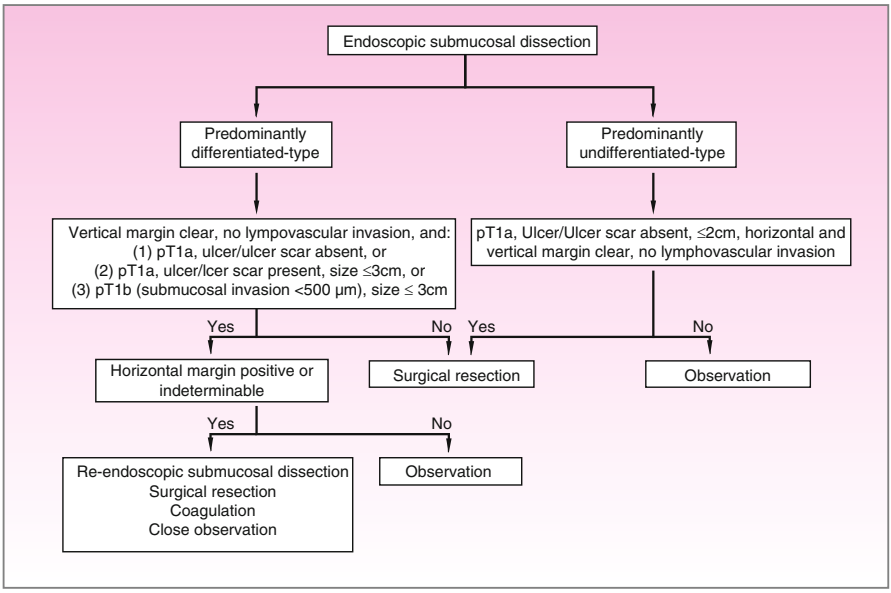

Fig. 16.4 Treatment options after endoscopic mucosal dissection. Modified from Japanese gastric cancer treatment guidelines 2014 [36]

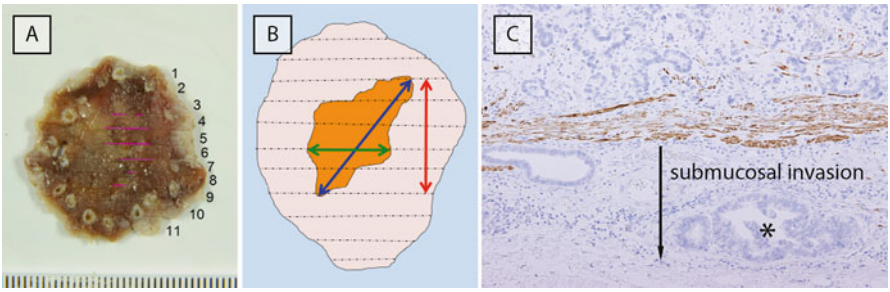

Fig. 16.5 Histopathologic workup of endoscopically resected specimen. (**a**) The distribution of tumor (*pink lines*) is mapped on photograph of the endoscopic resection specimen. (**b**) Measurement of the maximum tumor diameter of an endoscopically resected specimen. Estimation based on the section intervals (*red arrow*) or the largest tumor size on slides (*green arrow*) may result in underestimation compared with the true maximum diameter (*blue arrow*). (**c**) Immunohistochemistry for desmin (*brown* staining) helps to reproducibly measure the distance of submucosal invasion (see *arrow*), particularly in cases associated with fibrosis. *Asterisk* denotes tumor gland

Since the use of special stains significantly improves the detection sensitivity of lymphovascular invasion [41], we are now uniformly performing immunohistochemical staining using antipodoplanin (D2-40) antibody and elastic stains in lesions with submucosal invasion, even when the submucosal invasion is limited to minor areas.

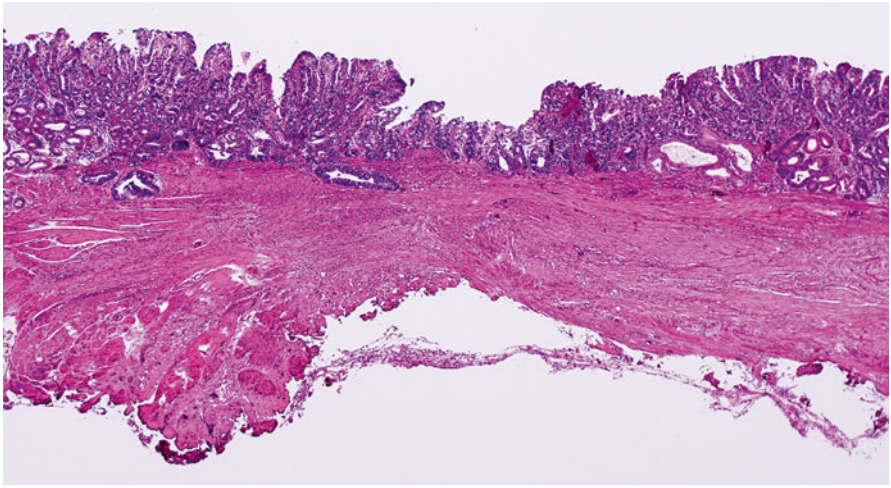

Fig. 16.6 Ulcer scar in an endoscopically resected specimen. Prominent submucosal fibrosis beneath differentiated-type adenocarcinoma

The presence of an ulcer scar not only compromises the endoscopic resection because of submucosal fibrosis, but this is also associated with an increased risk of lymph node metastasis in intramucosal gastric cancer (Fig. 16.6) [37, 40]. Therefore, the presence of an ulcer scar must be evaluated during the histopathological examination. Furthermore, a bona fide peptic ulcer scar must be distinguished from a biopsy scar, but in most instances the distinction is not difficult because the latter is associated with minimal submucosal fibrosis.

Among early gastric cancers, undifferentiated-type adenocarcinoma, including poorly differentiated adenocarcinoma and signet ring cell carcinoma, carry a higher risk of lymph node metastases than differentiated-type adenocarcinoma, which include tubular and papillary adenocarcinoma [6, 37, 39]. Accordingly, the Japanese treatment guidelines formulate more stringent criteria for the use of endoscopic resection as definitive treatment for undifferentiated-type adenocarcinoma [36]. The choice of treatment for "mixed differentiated and undifferentiated-type adenocarcinoma" (referred to as "mixed-type adenocarcinoma" hereafter) remains controversial. Interestingly, Hanaoka et al. analyzed a series of gastric cancers with submucosal invasion and showed that predominantly undifferentiated-type mixed-type carcinomas portend a high risk of lymph node metastasis, even when compared with pure undifferentiated-type carcinomas [39]. This result is somewhat paradoxical. However, if we assume that mixed-type adenocarcinomas develop through progression from differentiated-type adenocarcinomas, it would also be reasonable to assume that mixed-type adenocarcinomas represent more advanced lesions in terms of molecular abnormalities. These lesions may thus be more genetically diverse, which may explain increased biologic aggressive potential. Clearly, formulation of objective criteria for the curability assessment of mixed-type adenocarcinoma requires further study. Prospective studies assessing long-term outcome after endoscopic resection of mixed-type carcinomas are currently underway.

While not dealt with in the Japanese guidelines, the presence of a papillary adenocarcinoma component is also known to be an adverse prognostic factor. Papillary adenocarcinoma has a higher risk of lymphovascular involvement as well as lymph node and liver metastasis when compared to tubular adenocarcinoma [24, 42–44]. Another study focusing on endoscopically resected gastric cancers confirmed that the presence of a papillary adenocarcinoma component is an independent risk factor for lymphovascular invasion [45].

Helicobacter pylori–Negative Gastric Cancers

The pathogenic role of *H. pylori* in gastric carcinogenesis has been well established [3, 46]. Currently, *H. pylori*-negative gastric cancers are thought to constitute a very minor proportion of gastric cancer in Japan. Several studies from Japan reported the prevalence of *H. pylori*-negative gastric cancers to be less than 5 % [47–50]. However, it is often difficult to definitively rule out prior *H. pylori* infection. Matsuo et al. examined 3161 surgically resected gastric cancers for *H. pylori* infection status [49]. They analyzed histological as well as endoscopic findings to exclude previous infection and concluded that only 21 cases (0.66 %) did not display any evidence of current or prior *H. pylori* infection. Ono et al. analyzed a series of endoscopically resected early gastric cancers [50]. While current *H. pylori* infection was negative in 34 of 240 cases (14 %), after exclusion of cases associated with histological or endoscopic evidence of mucosal atrophy suggesting prior *H. pylori* gastritis, only one case (0.42 %) was thought to be unrelated to *H. pylori* infection.

These studies based on strict criteria suggest that *H. pylori*-unrelated gastric cancer is an extremely rare condition, likely responsible for <1 % of gastric cancers in Japan. Considering the declining *H. pylori* infection rate, particularly in younger generations, the prevalence of gastric cancer is expected to considerably decrease in the near future. This may also alter the histological spectrum of gastric cancer, because intramucosal signet ring cell carcinoma and adenocarcinoma of the fundic gland type have been suggested to be enriched among *H. pylori*-negative cases [51]. Conversely, since the vast majority of differentiated-type adenocarcinomas develop on a background of intestinalized mucosa, these will likely diminish following the decrease of *H. pylori* infection [52].

Many of the *H. pylori*-negative signet ring cell carcinomas are detected at an early stage and endoscopically appear as discolored lesions in the lower or middle part of the stomach in relatively young patients [49, 51]. Interestingly, Horiuchi et al. reported that most of these *H. pylori*-negative signet ring cell carcinomas were limited to the middle to superficial mucosal layer and showed lower proliferative activity than *H. pylori*-positive cases (Fig. 16.7a) [53]. The enrichment of early lesions may be explained in part because noninflamed background mucosa makes the detection of minute lesions somewhat less challenging as compared with chronically damaged mucosa associated with *H. pylori* infection. Another possibility is that *H. pylori*-negative signet ring cell carcinomas may naturally follow a more

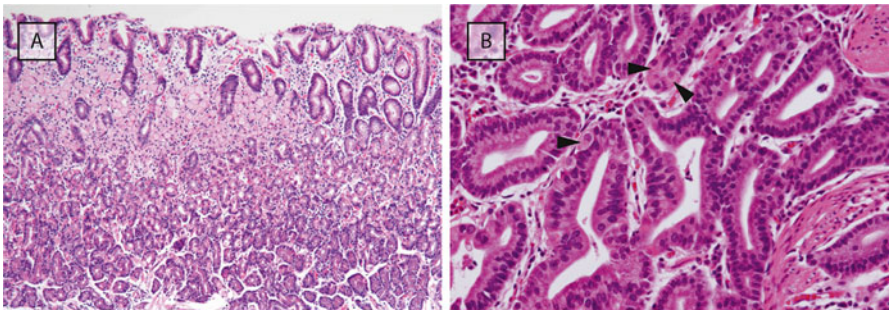

Fig. 16.7 *H. pylori*-negative signet ring cell carcinoma and adenocarcinoma of the fundic gland type. (**a**) Signet ring cells proliferate in the middle to surface layer of the nonatrophic fundic gland mucosa in *H. pylori*-negative signet ring cell carcinoma. (**b**) Adenocarcinoma of the fundic gland type shows proliferation of anastomosing glands composed of chief cells with abundant basophilic cytoplasm and smaller number of parietal cells with eosinophilic cytoplasm (*arrowheads*)

indolent clinical course, possibly because of the absence of the tumor-promoting effects of chronic inflammation.

H. pylori-negative signet ring cell carcinoma can also appear as a part of a cancer predisposition syndrome. Hereditary diffuse gastric cancer (HDGC), caused by germline *CDH1* mutations, was first reported in patients from three Maori families in 1998 [54]. Despite the high incidence of gastric cancer in general, HDGC had until very recently rarely been reported from East Asia. It remained unclear if this reflected the true rarity of this hereditary condition in this area. However, several cases of HDGC have now been reported from Japan [55–57]. This suggests that familial aggregation of diffuse gastric cancer may have been less apparent previously due to the high population incidence of sporadic gastric cancers in Japan. Conceivably, more HDGC cases may be diagnosed in the near future in parallel with the decrease of *H. pylori*-associated gastric cancers.

Adenocarcinoma of fundic gland type is a recently proposed entity, which develops in the nonatrophic fundic gland mucosa [58, 59]. These lesions are composed of chief cells and a smaller number of parietal cells, arranged in glands that frequently show an anastomosing pattern (Fig. 16.7b). These tumors usually present as small, early stage lesions and exhibit clinically indolent behavior. For this reason, there is controversy regarding the lesion's malignant potential as well as its proper nomenclature. Some authors have suggested that these lesions are preferably classified as adenoma in view of their clinically benign behavior [60]. However, several more recent studies have reported that at least a subset of these tumors exhibit frank submucosal invasion, lymphovascular invasion, and/or metastasis, implying biological heterogeneity of this entity [61–63]. Interestingly, these fundic gland-type neoplasms frequently harbor activating *GNAS* mutations, which are virtually absent in conventional adenocarcinoma, suggesting that these lesions also represent a genetically distinct group of tumors [64].

Concluding Remarks

In the near future the incidence of gastric cancer is expected to begin declining worldwide following the reducing *H. pylori* infection rate. However, at this point gastric cancer incidence remains high and is in fact increasing in Japan due to improved endoscopic detection techniques and increased life expectancy. The introduction of endoscopic resection has dramatically changed the therapeutic strategies for early gastric cancer. As a result, risk prediction of lymph node metastasis in endoscopically resected lesions has emerged as a new and major focus of clinicopathological research. A wealth of historic data on surgically resected material has contributed to the establishment of the current guidelines for endoscopic resection, and further research including prospective clinical studies will help us refine the curability assessment in endoscopically resected early gastric cancers.

Finally, the difference in histopathological classification of early gastric lesions between Japanese and Western pathologists remains a problematic issue. Although this does not pose problems in routine practice, it can be a major obstacle in research, in particular outside the field of pathology: it is unrealistic to expect basic researchers, endoscopists, and gastroenterologists to assimilate the different pathology classification systems. Attempts to minimize these discrepancies and define a uniform approach include the Vienna classification and the Padova classification [13, 22, 65], but this has not eliminated the problem. Further collaboration among centers across the globe is pivotal to formulate reproducible and widely acceptable classification criteria, which will serve as a basis for pathological, clinical, and basic researches.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359–86.
2. Asaka M, Kato M, Graham DY. Prevention of gastric cancer by Helicobacter pylori eradication. Intern Med. 2010;49:633–6.
3. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
4. Hirayama Y, Kawai T, Otaki J, Kawakami K, Harada Y. Prevalence of Helicobacter pylori infection with healthy subjects in Japan. J Gastroenterol Hepatol. 2014;29 Suppl 4:16–9.
5. Inoue M, Tsugane S. Epidemiology of gastric cancer in Japan. Postgrad Med J. 2005;81:419–24.
6. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer. 2011;14:101–12.
7. Murakami T. Pathomorphological diagnosis. Definition and gross classification of early gastric cancer. Gann Monogr Cancer Res. 1971;11:53–5.
8. Nishi M, Ishihara S, Nakajima T, Ohta K, Ohyama S, Ohta H. Chronological changes of characteristics of early gastric cancer and therapy: experience in the Cancer Institute Hospital of Tokyo, 1950-1994. J Cancer Res Clin Oncol. 1995;121:535–41.
9. Hamashima C, Shibuya D, Yamazaki H, Inoue K, Fukao A, Saito H, Sobue T. The Japanese guidelines for gastric cancer screening. Jpn J Clin Oncol. 2008;38:259–67.

10. Suzuki H, Gotoda T, Sasako M, Saito D. Detection of early gastric cancer: misunderstanding the role of mass screening. Gastric Cancer. 2006;9:315–9.
11. Lauwers G, Carneiro F, Graham D, Curado M, Franceschi S, Montgomery E, Tatematsu M, Hattori T. Gastric carcinoma. In: Bosman F, Carneiro F, Hruban R, Theise N, editors. WHO classification of tumors of the digestive system. 4th ed. Lyon: JARC; 2010.
12. Schlemper RJ, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, Stolte M, Watanabe H, Takahashi H, Fujita R. Differences in diagnostic criteria for gastric carcinoma between Japanese and western pathologists. Lancet. 1997;349:1725–9.
13. Schlemper RJ, Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Flejou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike M, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. Gut. 2000;47:251–5.
14. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma. 14th ed. Tokyo, Japan: Kanehara & CO., Ltd.; 2010. In Japanese.
15. Sakurai U, Lauwers GY, Vieth M, Sawabe M, Arai T, Yoshida T, Aida J, Takubo K. Gastric high-grade dysplasia can be associated with submucosal invasion: evaluation of its prevalence in a series of 121 endoscopically resected specimens. Am J Surg Pathol. 2014;38:1545–50.
16. Kolodziejczyk P, Yao T, Oya M, Nakamura S, Utsunomiya T, Ishikawa T, Tsuneyoshi M. Long-term follow-up study of patients with gastric adenomas with malignant transformation. An immunohistochemical and histochemical analysis. Cancer. 1994;74:2896–907.
17. Yamada H, Ikegami M, Shimoda T, Takagi N, Maruyama M. Long-term follow-up study of gastric adenoma/dysplasia. Endoscopy. 2004;36:390–6.
18. Kasuga A, Yamamoto Y, Fujisaki J, Okada K, Omae M, Ishiyama A, Hirasawa T, Chino A, Tsuchida T, Igarashi M, Hoshino E, Yamamoto N, Kawaguchi M, Fujita R. Clinical characterization of gastric lesions initially diagnosed as low-grade adenomas on forceps biopsy. Dig Endosc. 2012;24:331–8.
19. Matsubara A, Sekine S, Kushima R, Ogawa R, Taniguchi H, Tsuda H, Kanai Y. Frequent GNAS and KRAS mutations in pyloric gland adenoma of the stomach and duodenum. J Pathol. 2013;229:579–87.
20. Vieth M, Kushima R, Borchard F, Stolte M. Pyloric gland adenoma: a clinico-pathological analysis of 90 cases. Virchows Arch. 2003;442:317–21.
21. Vieth M, Kushima R, Mukaisho K, Sakai R, Kasami T, Hattori T. Immunohistochemical analysis of pyloric gland adenomas using a series of Mucin 2, Mucin 5AC, Mucin 6, CD10, Ki67 and p53. Virchows Arch. 2010;457:529–36.
22. Dixon MF. Gastrointestinal epithelial neoplasia: Vienna revisited. Gut. 2002;51:130–1.
23. Yao T, Utsunomiya T, Oya M, Nishiyama K, Tsuneyoshi M. Extremely well-differentiated adenocarcinoma of the stomach: clinicopathological and immunohistochemical features. World J Gastroenterol. 2006;12:2510–6.
24. Koseki K, Takizawa T, Koike M, Ito M, Nihei Z, Sugihara K. Distinction of differentiated type early gastric carcinoma with gastric type mucin expression. Cancer. 2000;89:724–32.
25. Saito A, Shimoda T, Nakanishi Y, Ochiai A, Toda G. Histologic heterogeneity and mucin phenotypic expression in early gastric cancer. Pathol Int. 2001;51:165–71.
26. Tajima Y, Yamazaki K, Makino R, Nishino N, Aoki S, Kato M, Morohara K, Kaetsu T, Kusano M. Gastric and intestinal phenotypic marker expression in early differentiated-type tumors of the stomach: clinicopathologic significance and genetic background. Clin Cancer Res. 2006;12:6469–79.
27. Endoh Y, Tamura G, Motoyama T, Ajioka Y, Watanabe H. Well-differentiated adenocarcinoma mimicking complete-type intestinal metaplasia in the stomach. Hum Pathol. 1999;30:826–32.
28. Okamoto N, Kawachi H, Yoshida T, Kitagaki K, Sekine M, Kojima K, Kawano T, Eishi Y. "Crawling-type" adenocarcinoma of the stomach: a distinct entity preceding poorly differentiated adenocarcinoma. Gastric Cancer. 2013;16:220–32.

29. Ushiku T, Arnason T, Ban S, Hishima T, Shimizu M, Fukayama M, Lauwers GY. Very well-differentiated gastric carcinoma of intestinal type: analysis of diagnostic criteria. Mod Pathol. 2013;26:1620–31.
30. Fukayama M, Ushiku T. Epstein-Barr virus-associated gastric carcinoma. Pathol Res Pract. 2011;207:529–37.
31. Uemura Y, Tokunaga M, Arikawa J, Yamamoto N, Hamasaki Y, Tanaka S, Sato E, Land CE. A unique morphology of Epstein-Barr virus-related early gastric carcinoma. Cancer Epidemiol Biomarkers Prev. 1994;3:607–11.
32. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.
33. Camargo MC, Kim WH, Chiaravalli AM, Kim KM, Corvalan AH, Matsuo K, Yu J, Sung JJ, Herrera-Goepfert R, Meneses-Gonzalez F, Kijima Y, Natsugoe S, Liao LM, Lissowska J, Kim S, Hu N, Gonzalez CA, Yatabe Y, Koriyama C, Hewitt SM, Akiba S, Gulley ML, Taylor PR, Rabkin CS. Improved survival of gastric cancer with tumour Epstein-Barr virus positivity: an international pooled analysis. Gut. 2014;63:236–43.
34. Gotoda T, Yamamoto H, Soetikno RM. Endoscopic submucosal dissection of early gastric cancer. J Gastroenterol. 2006;41:929–42.
35. Oka S, Tanaka S, Kaneko I, Mouri R, Hirata M, Kawamura T, Yoshihara M, Chayama K. Advantage of endoscopic submucosal dissection compared with EMR for early gastric cancer. Gastrointest Endosc. 2006;64:877–83.
36. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines. 4th ed. Tokyo, Japan: Kanehara & CO., Ltd.; 2014. In Japanese.
37. Gotoda T, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, Kato Y. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. Gastric Cancer. 2000;3:219–25.
38. Sekiguchi M, Suzuki H, Oda I, Abe S, Nonaka S, Yoshinaga S, Taniguchi H, Sekine S, Kushima R, Saito Y. Risk of recurrent gastric cancer after endoscopic resection with a positive lateral margin. Endoscopy. 2014;46:273–8.
39. Hanaoka N, Tanabe S, Mikami T, Okayasu I, Saigenji K. Mixed-histologic-type submucosal invasive gastric cancer as a risk factor for lymph node metastasis: feasibility of endoscopic submucosal dissection. Endoscopy. 2009;41:427–32.
40. Hirasawa T, Gotoda T, Miyata S, Kato Y, Shimoda T, Taniguchi H, Fujisaki J, Sano T, Yamaguchi T. Incidence of lymph node metastasis and the feasibility of endoscopic resection for undifferentiated-type early gastric cancer. Gastric Cancer. 2009;12:148–52.
41. Arigami T, Natsugoe S, Uenosono Y, Arima H, Mataki Y, Ehi K, Yanagida S, Ishigami S, Hokita S, Aikou T. Lymphatic invasion using D2-40 monoclonal antibody and its relationship to lymph node micrometastasis in pN0 gastric cancer. Br J Cancer. 2005;93:688–93.
42. Nishida T, Tanaka S, Haruma K, Yoshihara M, Sumii K, Kajiyama G. Histologic grade and cellular proliferation at the deepest invasive portion correlate with the high malignancy of submucosal invasive gastric carcinoma. Oncology. 1995;52:340–6.
43. Sekiguchi M, Kushima R, Oda I, Suzuki H, Taniguchi H, Sekine S, Fukagawa T, Katai H. Clinical significance of a papillary adenocarcinoma component in early gastric cancer: a single-center retrospective analysis of 628 surgically resected early gastric cancers. J Gastroenterol. 2015;50:424–34.
44. Yasuda K, Adachi Y, Shiraishi N, Maeo S, Kitano S. Papillary adenocarcinoma of the stomach. Gastric Cancer. 2000;3:33–8.
45. Sekiguchi M, Sekine S, Oda I, Nonaka S, Suzuki H, Yoshinaga S, Taniguchi H, Tsuda H, Kushima R, Saito Y. Risk factors for lymphatic and venous involvement in endoscopically resected gastric cancer. J Gastroenterol. 2013;48:706–12.
46. Correa P. Helicobacter pylori and gastric carcinogenesis. Am J Surg Pathol. 1995;19 Suppl 1:S37–43.
47. Kakinoki R, Kushima R, Matsubara A, Saito Y, Okabe H, Fujiyama Y, Hattori T. Re-evaluation of histogenesis of gastric carcinomas: a comparative histopathological study between Helicobacter pylori-negative and H. pylori-positive cases. Dig Dis Sci. 2009;54:614–20.

48. Kato S, Matsukura N, Tsukada K, Matsuda N, Mizoshita T, Tsukamoto T, Tatematsu M, Sugisaki Y, Naito Z, Tajiri T. Helicobacter pylori infection-negative gastric cancer in Japanese hospital patients: incidence and pathological characteristics. Cancer Sci. 2007;98:790–4.
49. Matsuo T, Ito M, Takata S, Tanaka S, Yoshihara M, Chayama K. Low prevalence of Helicobacter pylori-negative gastric cancer among Japanese. Helicobacter. 2011;16:415–9.
50. Ono S, Kato M, Suzuki M, Ishigaki S, Takahashi M, Haneda M, Mabe K, Shimizu Y. Frequency of Helicobacter pylori-negative gastric cancer and gastric mucosal atrophy in a Japanese endoscopic submucosal dissection series including histological, endoscopic and serological atrophy. Digestion. 2012;86:59–65.
51. Yamamoto Y, Fujisaki J, Omae M, Hirasawa T, Igarashi M. Helicobacter pylori-negative gastric cancer: characteristics and endoscopic findings. Dig Endosc. 2015;27:551–61.
52. Ohata H, Kitauchi S, Yoshimura N, Mugitani K, Iwane M, Nakamura H, Yoshikawa A, Yanaoka K, Arii K, Tamai H, Shimizu Y, Takeshita T, Mohara O, Ichinose M. Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer. Int J Cancer. 2004;109:138–43.
53. Horiuchi Y, Fujisaki J, Yamamoto N, Shimizu T, Miyamoto Y, Tomida H, Taniguchi C, Morishige K, Omae M, Ishiyama A, Yoshio T, Hirasawa T, Yamamoto Y, Tsuchida T, Igarashi M, Nakajima T, Takahashi H. Biological behavior of the intramucosal Helicobacter pylori-negative undifferentiated-type early gastric cancer: comparison with Helicobacter pylori-positive early gastric cancer. Gastric Cancer. 2016;19(1):160–5.
54. Guilford P, Hopkins J, Harraway J, Mcleod M, Mcleod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. Nature. 1998;392:402–5.
55. Sugimoto S, Yamada H, Takahashi M, Morohoshi Y, Yamaguchi N, Tsunoda Y, Hayashi H, Sugimura H, Komatsu H. Early-onset diffuse gastric cancer associated with a de novo large genomic deletion of CDH1 gene. Gastric Cancer. 2014;17:745–9.
56. Yamada H, Shinmura K, Ito H, Kasami M, Sasaki N, Shima H, Ikeda M, Tao H, Goto M, Ozawa T, Tsuneyoshi T, Tanioka F, Sugimura H. Germline alterations in the CDH1 gene in familial gastric cancer in the Japanese population. Cancer Sci. 2011;102:1782–8.
57. Yamada M, Fukagawa T, Nakajima T, Asada K, Sekine S, Yamashita S, Okochi-Takada E, Taniguchi H, Kushima R, Oda I, Saito Y, Ushijima T, Katai H. Hereditary diffuse gastric cancer in a Japanese family with a large deletion involving CDH1. Gastric Cancer. 2014;17:750–6.
58. Ueyama H, Matsumoto K, Nagahara A, Hayashi T, Yao T, Watanabe S. Gastric adenocarcinoma of the fundic gland type (chief cell predominant type). Endoscopy. 2014;46:153–7.
59. Ueyama H, Yao T, Nakashima Y, Hirakawa K, Oshiro Y, Hirahashi M, Iwashita A, Watanabe S. Gastric adenocarcinoma of fundic gland type (chief cell predominant type): proposal for a new entity of gastric adenocarcinoma. Am J Surg Pathol. 2010;34:609–19.
60. Singhi AD, Lazenby AJ, Montgomery EA. Gastric adenocarcinoma with chief cell differentiation: a proposal for reclassification as oxyntic gland polyp/adenoma. Am J Surg Pathol. 2012;36:1030–5.
61. Chan K, Brown IS, Kyle T, Lauwers GY, Kumarasinghe MP. Chief cell predominant gastric polyps: a series of 12 cases with literature review. Histopathology. 2016;68:825–33.
62. Fukatsu H, Miyoshi H, Ishiki K, Tamura M, Yao T. Gastric adenocarcinoma of fundic gland type (chief cell predominant type) treated with endoscopic aspiration mucosectomy. Dig Endosc. 2011;23:244–6.
63. Ueo T, Yonemasu H, Ishida T. Gastric adenocarcinoma of fundic gland type with unusual behavior. Dig Endosc. 2014;26:293–4.
64. Kushima R, Sekine S, Matsubara A, Taniguchi H, Ikegami M, Tsuda H. Gastric adenocarcinoma of the fundic gland type shares common genetic and phenotypic features with pyloric gland adenoma. Pathol Int. 2013;63:318–25.
65. Rugge M, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H. Gastric dysplasia: the Padova international classification. Am J Surg Pathol. 2000;24:167–76.

Chapter 17
Syndromic Gastric Polyps: At the Crossroads of Genetic and Environmental Cancer Predisposition

Lodewijk A.A. Brosens, Francis M. Giardiello, G. Johan Offerhaus, and Elizabeth A. Montgomery

Introduction

Gastric polyps are found in 1–4 % of all gastroscopies in the general population [1]. The vast majority are epithelial polyps. Other polypoid lesions, including neuroendrocrine tumors, pancreatic heterotopia, lymphoma, and mesenchymal polyps such as inflammatory fibroid polyp and gastrointestinal stromal tumor are relatively rare [2].

Mainly caused by differences in *Helicobacter pylori* (HP) infection, great geographical differences in prevalence of different gastric polyps are observed. In general, hyperplastic polyps and adenomas are much more prevalent in countries with high rates of HP infection. In Western countries, with low prevalence of HP infection,

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fundic gland polyps (FGP) are the most common polyp type comprising up to 77 % of all gastric polyps [1, 2]. Fundic gland polyps are usually <0.5 cm and characterized by cystically dilated oxyntic glands lined by parietal and chief cells. Sporadic FGPs are usually single or few in number but can be numerous in patients using proton pump inhibitors (PPI). Dysplasia in sporadic fundic polyps is very rare. Multiple fundic gland polyps (polyposis) are a frequent manifestation of familial adenomatous polyposis (FAP) and in this context low-grade dysplasia is often seen, although the risk of malignant progression is low.

Hyperplasic polyps, the second most common type of gastric polyps in Western populations, comprise almost 15 % of all gastric polyps [2]. Hyperplastic polyps are a hyperproliferative response to tissue injury and typically occur in patients with *Helicobacter pylori* or autoimmune chronic gastritis, atrophy and intestinal metaplasia. In addition, mucosal prolapse can result in hyperplastic-type polyps [3]. Gastric hyperplastic polyps are often multiple and most frequently found in the antrum. These polyps vary from 0.5 to 1.5 cm, but can be very large causing gastric obstruction. Gastric hyperplastic polyps are characterized by hyperplastic, dilated, elongated, distorted, and branching foveolae with edematous stroma, lined by reactive foveolar epithelium. Large polyps may become eroded and cause chronic blood loss and iron deficiency anemia. Intestinal metaplasia as well as dysplasia has been reported in 1–20 % of these polyps [1, 4].

Gastric adenomas are the third most common type of polyp in the Western population, but only account for <1 % of all gastric polyps [2]. Histologically, gastric foveolar-type adenoma, intestinal-type adenoma, and pyloric gland adenoma can be distinguished [5, 6]. Whereas background gastric mucosa in gastric foveolar-type adenomas is usually normal, intestinal-type adenomas typically occur in a background of *Helicobacter pylori* infection, chronic gastritis, atrophy, and intestinal metaplasia. The more recently recognized pyloric gland adenoma (PGA) is characterized by densely packed cuboidal to low columnar epithelium resembling pyloric gland cells and typically occurs in association with autoimmune atrophic gastritis. Moreover, activating mutation of *GNAS* seems to be specific for this type of adenoma since it was found in 65 % of PGAs but not in gastric foveolar-type or intestinal-type adenomas [7]. Recently, PGAs were also described in FAP and Lynch syndrome, although the lesions reported in Lynch syndrome arose in a population that differed from the one associated with FAP [8, 9]. Both intestinal-type adenomas and PGAs carry a higher risk of neoplastic progression than gastric-foveolar type adenomas, which typically harbor low-grade dysplasia [5, 6].

True hamartomatous polyps in the stomach are rare and exclusively occur in the setting of hamartomatous polyposis syndromes. However, as opposed to colonic hamartomatous polyps, gastric hamartomatous polys lack specific histology and are difficult, if not impossible, to differentiate from gastric hyperplastic polyps. Clinical context is essential to make a diagnosis of a gastric hamartomatous polyp [10].

Several recent studies shed new light on the pathology of gastric polyps occurring in the setting of gastrointestinal polyposis and other syndromes (Table 17.1). Although morphology of gastric polyps is less specific than of colonic polyps, in particular in the case of hamartomatous polyposis syndromes, knowledge of polyp

Table 17.1 Syndromes associated with gastric polyps

Syndrome	Genes	Mode of inheritance	Incidence of gastric polyps	Number of gastric polyps	Histological type of gastric polyps	Gastric cancer lifetime risk
(Attenuated) familial adenomatous polyposis	*APC*	Autosomal dominant	>66 % [8]	Multiple	FGP (predominant), gastric foveolar adenomas, pyloric gland adenoma	Not increased in Western patients [27] (<1 %)
MUTYH-associated polyposis	*MUTYH (MYH)*	Autosomal recessive	10–30 % [32, 33]	Unclear	FGPs (predominant), adenomas	Not increased [14]
Lynch syndrome	*MLH1, MSH2, MSH6, PMS2,* or *EpCAM*	Autosomal dominant	Rare	Few	Pyloric gland adenoma	5–8 % [12]
Peutz–Jeghers syndrome	*LKB1 (STK11)*	Autosomal dominant	25 % [40]	Few to numerous	Hamartomatous polyps	29 % [42]
Juvenile polyposis syndrome	*SMAD4* (~30 %) *BMPR1A* (~20 %)	Autosomal dominant	60–83 % [55, 56]	Few to numerous	Hamartomatous polyps	11–21 % [46, 52, 58]
Cowden (PTEN hamartoma tumor syndromes)	*PTEN*	Autosomal dominant	Up to 100 % [66, 67]	Few to multiple	Hamartomatous and hyperplastic polyps	Not increased as far as known
Gastric adenocarcinoma and proximal polyposis of the stomach	Unknown	Autosomal dominant	100 % [70]	Numerous (polyposis)	FGPs (primarily). Few hyperplastic polyps and adenomas	Unknown
Neurofibromatosis type 1	*NF1*	Autosomal dominant	<2 % [75]	1–3 (Mostly) to multiple	Inflammatory/hyperplastic polyps	Not increased
McCune–Albright syndrome	*GNAS*	Noninherited postzygotic mutation	unknown	2 to multiple	Hamartomatous polyps, FGP, hyperplastic, adenoma	Not increased
Cronkhite–Canada syndrome	n/a	Non inherited	100 %	Numerous	Inflammatory/hamartomatous polyps	Not increased

FGP fundic gland polyp

n/a: not applicable

types occurring in different syndromes may help to recognize a patient with syndromic polyps. In addition, studying tumorigenesis in hereditary syndromes can increase our understanding of gastric tumorigenesis in general.

This chapter will review the prevalence, histopathology, and genetics of gastric polyps in the well-established gastrointestinal polyposis syndromes. In addition, the recently recognized hereditary syndrome Gastric Adenocarcinoma and Proximal Polyposis of the Stomach (GAPPS), and gastric polyps in other not primarily gastrointestinal syndromes such as McCune–Albright syndrome and neurofibromatosis type 1, will be discussed.

Familial Adenomatous Polyposis Syndrome

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome caused by germline mutation in the *Adenomatous Polyposis Coli* (*APC*) gene. Classic FAP is characterized by development in teenage years of hundreds to thousands adenomatous polyps (≥100) throughout the colorectum. About 50 % of patients develop adenomas by age 15 and 95 % by age 35. If left untreated, CRC is diagnosed at an average age of 39 years (range 35–43 year) [11, 12]. Attenuated FAP (AFAP) is defined by the presence of oligopolyposis; on average patients have 30 polyps. The diagnosis should be considered in patients 40–50 year old with 10–99 adenomas cumulatively. Patients with AFAP have a 70 % lifetime risk of CRC, presenting about 12 years later than classic FAP. Of note, oligopolyposis can be an expression of a germline *APC* mutation or a biallelic *MUTYH* mutation (see next section) [12–14].

A variety of benign and malignant extracolonic manifestations have been described in FAP, of which duodenal adenomas and cancer and desmoids tumors are clinically now the most challenging [12, 15]. Duodenal adenomas are present in 30–70 % of FAP patients with a lifetime risk of almost 100 %. The lifetime risk of duodenal adenocarcinoma is 4–10 % [12].

More than two-thirds of FAP patients have gastric polyps [8, 16] (Fig. 17.1). The vast majority (nearly 80 %) of gastric polyps in FAP are benign fundic gland polyps occurring in the gastric fundus and body [8]. Typically, FAP patients have multiple FGPs presenting as gastric polyposis [17]. Of note, sporadic fundic gland polyposis can occur, but this phenomenon is always associated with PPI, as are most single sporadic fundic gland polyps. Also, both sporadic single FGPs and sporadic fundic gland polyposis rarely show dysplasia, harbor *β-catenin* mutations, lack *APC* mutations, and never progress to cancer [18–20].

FGPs vary in size from a few millimeters to a centimeter. Microscopically, these polyps have dilatation and cystic changes of the fundic glands. Low-grade dysplasia is present in up to a third of FGPs in FAP, but high-grade dysplasia and malignant transformation in FGPs in FAP is exceedingly rare [8, 21, 22]. Genetically, fundic gland polyps in FAP frequently show a somatic second hit inactivation of the wild-type *APC* allele that precedes dysplasia [23]. In contrast, sporadic FGPs without

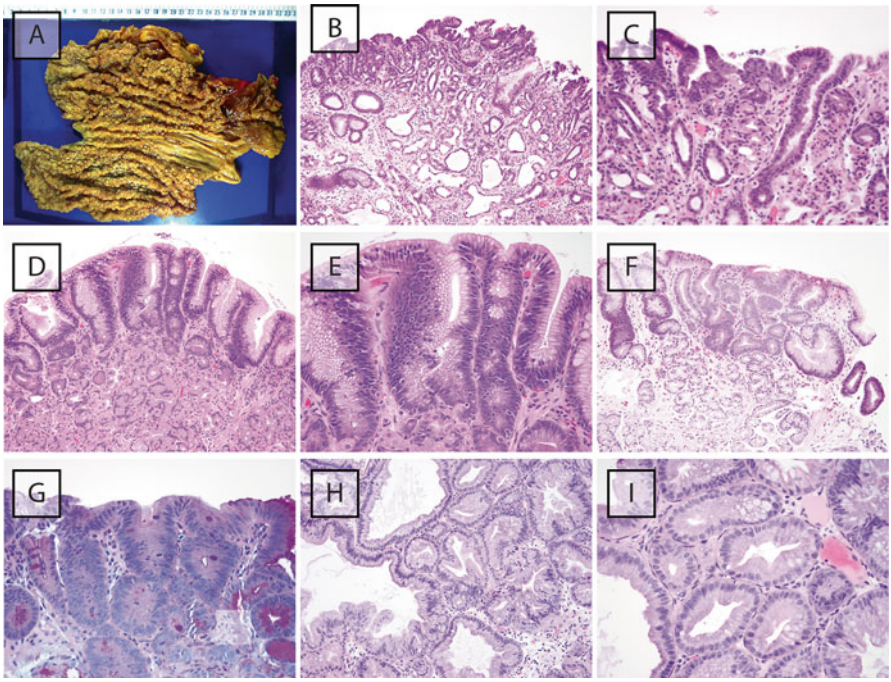

Fig. 17.1 (**a**) Familial adenomatous polyposis, gross specimen. Numerous fundic gland polyps and gastric foveolar-type adenomas were identified. (**b**) Familial adenomatous polyposis, fundic gland polyp with surface low-grade foveolar-type dysplasia. Such lesions are unlikely to progress to carcinoma. Note the dilated oxyntic glands beneath the surface. (**c**) Familial adenomatous polyposis, fundic gland polyp with surface low-grade foveolar-type dysplasia. This is a higher magnification of the lesion depicted in (**b**). (**d**) Gastric adenoma, gastric foveolar type in familial adenomatous polyposis. This lesion is from a Western patient and lacks background gastritis. (**e**) Gastric adenoma, gastric foveolar type in familial adenomatous polyposis. At high magnification note that each surface cell has a droplet like well marginated neutral mucin cap. This is a higher magnification of the lesion depicted in (**d**). F. Gastric adenoma, intestinal type in familial adenomatous polyposis. This lesion arose in the antrum but note that there is no background intestinal metaplasia such that this lesion, like gastric foveolar adenomas in FAP patients, is more akin to a sporadic colorectal adenoma as opposed to a colitis-associated dysplastic polypoid lesion. (**g**) Gastric adenoma, intestinal type in familial adenomatous polyposis, PAS/AB stain. Note the goblet cells within the lesion. (**h**) Pyloric gland adenoma in familial adenomatous polyposis. These lesions usually arise in the setting of pyloric metaplasia in the sporadic setting but in FAP they arise in uninflamed oxyntic mucosa. Note the ground glass cytoplasm and compare it to the appearance of the cytoplasm of the foveolar adenoma in (**e**). I. Pyloric gland adenoma in familial adenomatous polyposis. The nuclei are round and form a monolayer

dysplasia typically show a single *β-catenin* mutation. Although sporadic FGPs with dysplasia are extremely rare they often harbor an *APC* mutation [19, 24].

Up to 20 % of gastric polyps in Western FAP patients are adenomas [8]. Most of these (85 %) occur in the gastric body and are gastric foveolar-type adenomas with low-grade dysplasia. Distinction between a FGP with low-grade dysplasia and a gastric foveolar-type adenoma can be difficult. Background gastric mucosa in FAP

patients with gastric foveolar-type adenomas is typically normal. This type of adenoma, therefore, seems to represent an isolated lesion caused by the germline *APC* mutation, similar to colonic adenomas in FAP. Gastric foveolar-type adenomas have a low risk of progression to high-grade dysplasia [5].

About 2–3 % of all gastric polyps (14 % of all adenomas) in FAP are classified as pyloric gland adenomas. These polyps are characterized by densely packed cuboidal to low columnar epithelium with round nuclei without prominent nucleoli and pale or eosinophilic, "ground glass" cytoplasm, resembling pyloric gland cells. PGAs are found in the gastric body and fundus and high-grade dysplasia is seen in about 10–15 % [6]. In the general population, PGAs are typically found in association with autoimmune atrophic gastritis and pseudopyloric metaplasia (or antralization) although even in this setting PGAs are still very rare with a prevalence of just 1 % and comprise 15 % of all gastric adenomas (the vast majority being intestinal-type adenomas) [25]. Interestingly, PGAs in FAP occur in undamaged background gastric mucosa but seem to be more prevalent in FAP than in autoimmune atrophic gastritis since PGAs were found in 6 % of FAP patients [6, 8].

Intestinal-type adenomas are very rare in Western FAP patients (1–2 % of gastric adenomas in FAP) [8]. Interestingly, a higher prevalence of gastric adenomas of 40–50 % has been found in Asian FAP patients, compared to 10–20 % in Western patients. This higher prevalence is likely caused by more widespread *Helicobacter pylori* infection and chronic atrophic gastritis and intestinal-type adenomas. Indeed, a strong correlation between presence of gastric adenomas (subtype not specified) and *Helicobacter pylori* associated atrophic gastritis has been shown in Japanese FAP patients [26].

Western FAP patients do not carry an increased risk of gastric cancer, which is consistent with the low malignant potential of the gastric-foveolar-type adenomas seen in most Western FAP patients [27]. In contrast, a 3–4 times higher risk of gastric cancer is seen in FAP patients in Asian countries where gastric cancer is also more prevalent in the general population. This difference is best explained by higher prevalence of *Helicobacter pylori* infection and associated atrophic gastritis and intestinal metaplasia in these populations [28, 29]. Therefore, presence of *Helicobacter pylori* infection, gastric atrophy, and intestinal-type adenomas seem to be particularly important to identify FAP patients at increased risk of gastric cancer [30]. Also, presence of pyloric gland adenomas in FAP may be relevant for an increased cancer risk, but this needs further studies. Gastric foveolar-type dysplasia, as long as it is low grade, may be ignored with regard to an increased risk of gastric cancer.

Screening for upper gastrointestinal tumors in Western FAP is thus mainly directed at duodenal/ampullary adenomas and cancer. Screening endoscopy should start at age 25–30 years and be repeated every 0.5–4 years depending on Spigelman stage of duodenal polyposis [15]. Examination of the stomach should include random sampling of fundic gland polyps and larger suspicious looking polyps with biopsies from surrounding flat mucosa. Gastric surgery should be reserved for high-grade dysplasia or cancer [12].

MUTYH-Associated Polyposis

MUTYH-associated polyposis (MAP) is an autosomal recessive disorder caused by biallelic germline mutation in the *MUTYH* gene [14]. Most patients have between 10 to a few hundred colorectal adenomas, mimicking the colonic phenotype of attenuated FAP. However, MAP can also present with early onset CRC with few to zero polyps [14]. Most colorectal polyps in MAP are adenomas, but serrated polyps can occur [31]. The risk of CRC in MAP is 19 % by age 50 and 43 % by age 60. The average age of CRC onset is 48 years. Relatives of MAP patients with a heterozygous *MUTYH* mutation have a risk of CRC comparable to that of first-degree relatives of patients with sporadic CRC [14]. Compared to the general population, MAP patients have an almost doubled risk of extraintestinal malignancies, including ovarian, bladder, skin, and possibly breast cancer [32].

Gastric polyps are found in about 10–33 % of MAP patients and mainly include fundic gland polyps, sometimes with low-grade dysplasia, and less frequently pure adenomas [32, 33]. The histological spectrum of polyps in MAP is thus similar to FAP. Analogously, there seems to be no increased risk of gastric cancer in MAO, but an increased lifetime risk of duodenal cancer of about 4 % [14, 32].

Lynch Syndrome

Lynch syndrome (LS) (also known as hereditary nonpolyposis colorectal cancer; HNPCC) is an autosomal dominantly inherited syndrome caused by germline mutation in one of the mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) or the *EpCAM* gene [11]. Colorectal cancer is the major clinical consequence of LS with a lifetime risk as high as 75 %, depending on the mismatch repair (MMR) gene mutated [12]. The average age of CRC diagnosis in LS patients is 44–61 years compared to 69 years in sporadic CRC. The histopathology of LS colorectal cancer is often poorly differentiated, with signet cell histology, abundant extracellular mucin, tumor infiltrating lymphocytes, and a Crohn's like lymphoid host response to tumor [34].

In addition to colorectal cancer, patients with Lynch syndrome have an increased risk for many extracolonic malignancies. Endometrial cancer is the second most common malignancy in LS patients with a lifetime risk between 15 and 71 %, depending on the specific MMR gene mutation. Other neoplasms with lifetime risks ranging from 4 to 25 % are urothelial cell carcinoma; adenocarcinomas of the ovary, stomach, hepatobiliary tract, and small bowel; brain cancer (glioblastoma); and cutaneous sebaceous neoplasms. The lifetime risk of gastric cancer is about 5 % in female and 8 % in male LS patients [12]. As in colorectal carcinomas, gastric carcinomas in LS are characterized by microsatellite instability and are usually of intestinal type [9, 35, 36].

Compared to patients with attenuated FAP or MAP, LS patients develop few colorectal adenomas (usually <3 adenomas). But the adenoma–carcinoma sequence appears accelerated in LS with polyp to cancer intervals estimated at 35 months compared to 10–15 years in sporadic cancer [37]. Also gastric polyps are rare which complicates surveillance strategies for gastric cancer [38]. A recent study showed MSI and loss of MMR protein expression in pyloric gland adenomas in 3 of 15 patients with LS and gastric cancer suggesting that pyloric gland adenomas may be a precursor to gastric cancer in LS [9]. However, the vast majority of patients in this study had atrophic gastritis and intestinal metaplasia and, based on the illustrations in this article, the reported lesions may have not have wholly conformed to pyloric gland adenomas as initially described [6]. Further studies are needed to confirm this observation. Better understanding of gastric cancer carcinogenesis and identification of biomarkers or precursor lesions may lead to more effective screening methods to prevent gastric cancer in LS.

Peutz–Jeghers Syndrome

Peutz–Jeghers syndrome (PJS) is an autosomal dominant syndrome caused by germline mutation of the *LKB1/STK11* gene. PJS is characterized by gastrointestinal polyposis, perioral pigmentation, and a moderate or high risk of a diversity of malignancies with an overall lifetime risk of any cancer of 81 % by age 70 [12, 39]. Patients are particularly at increased risk for gastrointestinal malignancies, including colorectal, gastric, small bowel, and pancreatic cancer. In addition, increased risk for a variety of extraintestinal malignancies exists, including lung, breast, and gynecological cancer [12].

Polyps most frequently occur in the small intestine in about 95 % of PJS patients. The colon and stomach each are affected in about 25 % of patients [40]. Guidelines recommend that upper and lower gastrointestinal endoscopies are performed first at age 8 years and repeated at least every 3 years when polyps are found. Small bowel imaging should be done at least every 3 years starting at age 8 years. Clearing of all polyps is recommended when possible [12].

Small bowel and colon polyps are typically pedunculated, whereas, gastric polyps are often sessile. Polyps vary in size from 0.1 to 5 cm. These large polyps make patients prone to small bowel intussusception [12].

Colorectal Peutz–Jeghers polyps have a distinct morphology characterized by villous architecture and arborizing smooth muscle. Also, characteristic lobulated clusters of colonic crypts have been described in syndromic PJS polyps which may discriminate them from other polyps such as hyperplastic, prolapse type, or juvenile polyps [41]. In contrast, gastric Peutz–Jeghers polyps are difficult to distinguish from gastric juvenile or hyperplastic polyps [10]. Sometimes, lobulated clustering of gastric foveolae/pits can be seen (Fig. 17.2), but the diagnostic value of this finding in the stomach has not yet been addressed. Without knowledge of the clinical

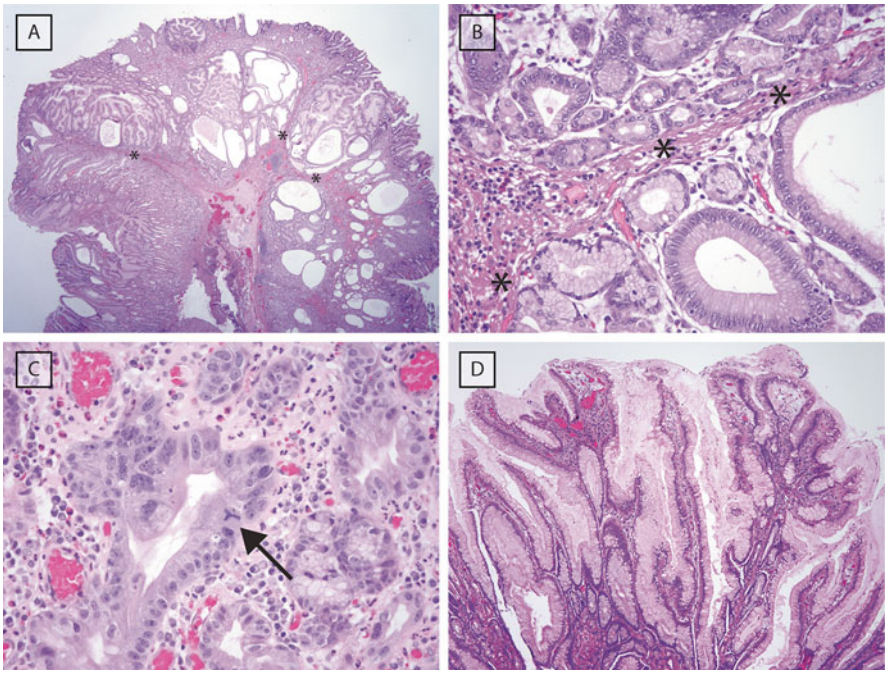

Fig. 17.2 (**a**) Gastric Peutz–Jeghers polyp. Note the cords of smooth muscle that partition the lesion into sections (asterisks). In a large sample such as this, it can be possible to prospectively suggest the diagnosis but in superficial samples, it can be impossible to separate these from hyperplastic polyps. (**b**) Gastric Peutz–Jeghers polyp. Note the strand of smooth muscle in the center of the field (asterisks). (**c**) Gastric Peutz–Jeghers polyp. Dysplasia is rare in such polyps but is encountered. This example of high-grade dysplasia arose in a patient with many gastric Peutz–Jeghers polyps. Note the atypical mitotic figure (triaster mitosis) (*arrow*). (**d**) Gastric Peutz–Jeghers polyp. This polyp arose in a patient with known Peutz–Jeghers syndrome and normal flat gastric mucosa. In isolation it is not possible to separate it from a hyperplastic polyp. A clue is the smooth surface

context one should be cautious when gastric polyps are used to establish a new diagnosis of PJS [10]. Additional endoscopic examination and sampling of polyps in the small intestine or colon, as well as genetic testing should be recommended.

PJS is associated with a lifetime risk of gastric cancer of 29 % [42, 43]. Whether the Peutz–Jeghers polyp is the actual precursor to gastrointestinal cancer is a matter of debate. Dysplasia in a Peutz–Jeghers polyp is very rare (Fig. 17.2). It has been suggested that Peutz–Jeghers polyps are in fact an epiphenomenon to the cancer prone condition and not obligate malignant precursors [44]. Studies in the colon show a protracted clonal evolution in normal colonic crypts from PJS patients. This allows a greater number of mutations to be retained in the crypt which accelerates somatic evolution and can explain the increased risk of colorectal cancer in PJS. Pretumor progression has not yet been studied in gastric mucosa of PJS patients [45].

Juvenile Polyposis Syndrome

Juvenile polyposis syndrome (JPS) is an autosomal dominant disorder characterized by multiple juvenile polyps primarily in the colorectum but also elsewhere in the gastrointestinal tract. Juvenile polyposis syndrome is diagnosed in patients with five or more juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract, or any number of juvenile polyps and a positive family history of juvenile polyposis [46]. A germline mutation in the *SMAD4* or *BMPR1A* gene can be identified in about 50–60 % of JPS patients [47]. A variant of JPS (juvenile polyposis of childhood) which shows phenotypic overlap with PTEN hamartoma syndromes (next section) is caused by contiguous deletion of the *BMPR1A* and *PTEN* genes which are both located on chromosome 10q23 [46, 48–50].

Patients with JPS have an increased risk of several gastrointestinal malignancies. The lifetime risk of colorectal cancer has been calculated to be 38 % but may be as high as 70 % [46, 51]. In addition, JPS patients appear to be at increased risk of stomach, duodenal, and pancreatic cancer, but no formal risk analysis for these malignancies exists [52]. Evaluation of literature reports suggests that gastric and small bowel carcinoma, together, occur at about one-fifth the frequency of colorectal cancers in this patient group [51]. *SMAD4* mutations are associated with a more aggressive gastrointestinal phenotype, involving higher incidence of colonic adenomas and carcinomas and more frequent upper gastrointestinal polyps and gastric cancer [53, 54].

Polyps in JPS predominantly occur in the colorectum, varying in number from five to several hundreds. In addition, polyps can be found in the stomach (Fig. 17.3), duodenum, jejunum, and ileum, but few studies examined upper gastrointestinal tract involvement in juvenile polyposis systematically [55–58]. The incidence of gastric polyps in JPS varies between 60 and 85 % and the incidence of duodenal polyps between 14 and 33 % [55, 57, 58]. JPS patients with a germline *SMAD4* mutation seem to have more severe gastric polyposis than patients with a *BMPR1A* mutation or those with no germline mutation identified [53, 59, 60]. One study found a significantly higher frequency of gastric polyposis in *SMAD4* mutations carriers (73 %) than BMPR1A mutation carriers (8 %) and all seven cases of gastric cancer occurred in families with *SMAD4* mutations [54].

A recent study evaluated upper gastrointestinal tract pathology in 41 juvenile polyposis patients. Twenty-two patients had upper gastrointestinal endoscopy with biopsies and 13 of these patients (59 %) developed gastric polyps. Almost 25 % of patients had profuse gastric polyposis for which gastrectomy or long-term parenteral feeding was indicated [56]. The youngest patient diagnosed with gastric polyps was 7 years old and had a *SMAD4* mutation. Gastric polyp morphology was characterized by irregular hyperplastic glands mostly lined by foveolar epithelium (Fig. 17.4). Dysplasia was found in 8 of 56 gastric juvenile polyp biopsies (14 %) in two patients, four of which contained high-grade dysplasia. Two of the polyps with dysplasia showed intestinal differentiation, and 6 showed both intestinal and pyloric gland differentiation, intermixed with foveolar epithelium. Pyloric gland differentiation was only observed in gastric juvenile polyps with dysplasia, whereas intestinal

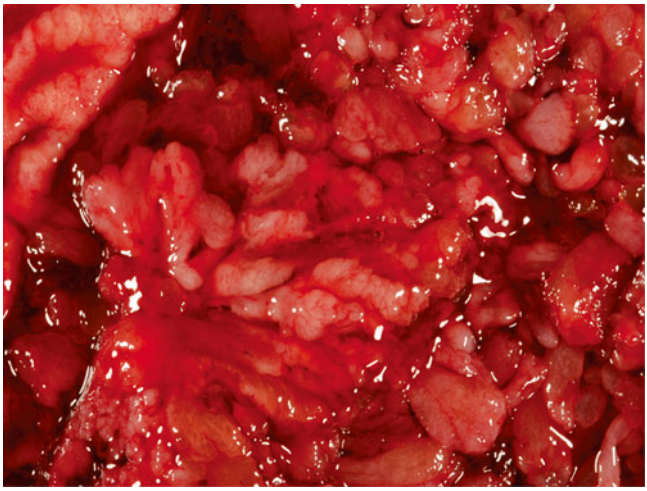

Fig. 17.3 Gastric juvenile polyposis, gastrectomy specimen, macroscopic. The stomach is carpeted with polyps in this case. One of them contained a carcinoma

differentiation was also seen in four gastric JP without dysplasia. However, the vast majority of gastric juvenile polyps only showed foveolar epithelium without dysplasia (44 of 56) with unremarkable background mucosa [56]. High-grade dysplasia and gastric cancer arise in gastric juvenile polyps and these polyps are thus bona fide precursor lesion for gastric cancer in JPS [61].

Importantly, distinction between gastric hyperplasic, juvenile, or Peutz–Jeghers polyps is difficult or impossible. Without knowledge of the clinical context the pathologist should be cautious to call a gastric polyp a juvenile polyp. Rather it is advisable to use a broader term such as hamartomatous polyp, not otherwise specified [10, 55]. Additional endoscopic examination and sampling of polyps in the small intestine or colon, as well as genetic testing are recommended [10]. In this regard, however, loss of SMAD4 immunostaining in colonic juvenile polyps is specific for an underlying *SMAD4* germline mutation and can be used as an adjunct in the molecular diagnosis of JPS [62]. Although the value of SMAD4 loss in gastric juvenile polyps has not been investigated, we have observed loss of SMAD4 protein expression in gastric polyps from a patient with JPS and a germline *SMAD4* mutation (Fig. 17.4).

To conclude, gastric polyps occur in 60 and 85 % of patients with JPS, can develop at young age, and can be profuse requiring parenteral feeding or gastrectomy. In addition, dysplasia can be found in almost 15 % of gastric polyps in JPS and patients seem to be at increased risk of gastric cancer, although the exact magnitude is unclear. Upper gastrointestinal tract endoscopy is, therefore, strongly recommended for patients with JPS. Current guidelines recommend upper endoscopy with removal of polyps >5 mm, starting at age 12 or earlier in case of symptoms and should be repeated every 1–3 years, depending on the severity of upper gastrointestinal polyposis [12].

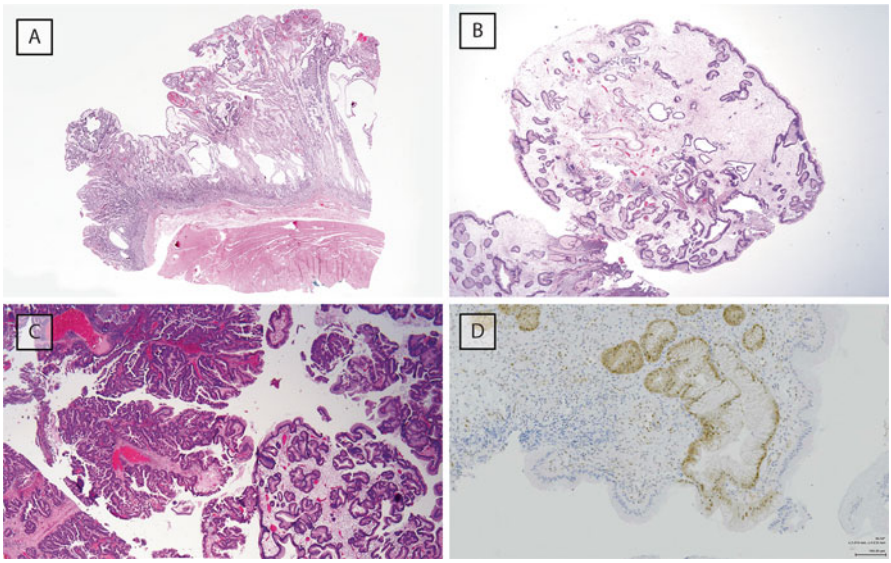

Fig. 17.4 (**a**) Gastric juvenile polyposis, gastrectomy specimen, low magnification. Note the cystically dilated glands. Additionally, one can imagine that a superficial biopsy of any of the lesions would be impossible to distinguish from a hyperplastic polyp. (**b**) Gastric juvenile polyposis, endoscopic biopsy. The prominent lamina propria edema and the smooth surface are clues that this is a juvenile polyp rather than a hyperplastic polyp. (**c**) Gastric juvenile polyposis with dysplasia. The nondysplastic component is present in the right half of the image whereas the left half shows extensive dysplasia. (**d**) SMAD4 immunohistochemistry in a gastric juvenile polyp showing loss of SMAD4 immunostaining consistent with the presence of a germline *SMAD4* mutation in this patient with juvenile polyposis syndrome

Cowden Syndrome (PTEN Hamartoma Tumor Syndrome)

PTEN hamartoma tumor syndrome (PHTS) is a heterogeneous group of autosomal dominant disorders caused by germline mutation of *PTEN* gene and includes Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome (BRRS), and Proteus syndrome. Most PHTS cases correspond to Cowden syndrome and the terms are often used interchangeable. Clinical features of CS include mucocutaneous lesions (facial trichilemmoma, acral keratoses, papillomatous papules and mucosal lesions are pathognomic) increased risks for malignancies (breast, thyroid, endometrial, colorectal, kidney, and melanoma) as well as benign hamartomatous overgrowth of tissues, including gastrointestinal polyposis, and macrocephaly. The lifetime risk of colorectal cancer is about 10–15 % [12]. The primary clinical features of BRRS include macrocephaly, hamartomatous intestinal polyps, lipomas, and pigmented macules on the penis [63].

Gastrointestinal polyps are almost universally present in CS patients. Polyps in CS occur throughout the entire gastrointestinal tract and typically represent a mixture of histologies [64]. In the colon hyperplastic polyps, hamartomatous/juvenile

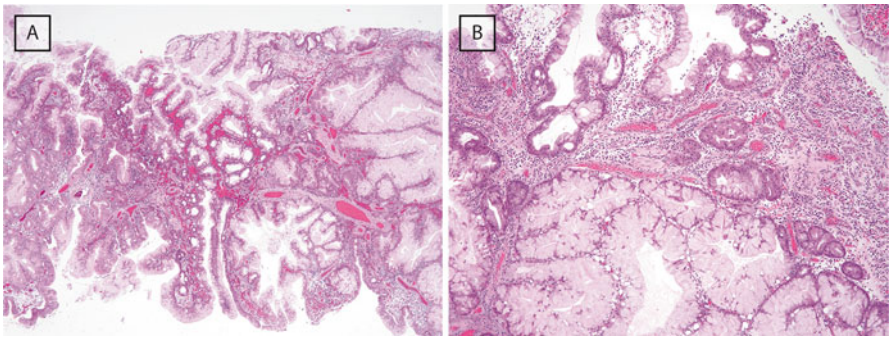

Fig. 17.5 (**a**) Gastric polyp from a patient with Cowden's syndrome. The appearances are similar to those seen in polyps in the setting of juvenile polyposis and similarly difficult to distinguish from those of hyperplastic polyps. (**b**) Gastric polyp from a patient with Cowden's syndrome. Note the hyperplastic appearing foveolar type epithelium and disorganized glands

polyps, adenomas, ganglioneuromas, and lymphoid follicles are most frequent [65]. Of note, colonic hyperplastic polyps are not considered part of the diagnostic criteria of PHTS [63]. Duodenal polyps are mainly hamartomas and some ganglioneuromas and adenomas [65]. More than 80 % of CS patients have diffuse esophageal glycogenic acanthosis which, in combination with colonic polyposis, may be diagnostic for CS [63, 66, 67].

Gastric polyps are usually numerous and can be found in almost all patients with Cowden syndrome (Fig. 17.5) [66, 67]. The polyps range in size from 0.1 to 2 cm. Histologically most are diagnosed as hyperplastic or hamartomatous [66, 67]. Dysplasia has not been reported in gastric polyps in Cowden syndrome. Nevertheless, a few cases of gastric cancer have been reported in this syndrome, but no formal risk analysis exists that shows an increased risk [64, 68, 69]. Interestingly, two of these gastric cancers were poorly differentiated or of signet cell histology, and one patient presented with two synchronous gastric carcinomas [64, 68].

The diversity of gastrointestinal polyps, including hamartomatous and ganglioneuromatous polyps and diffuse glycogen acanthosis in the esophagus suggests the diagnosis of Cowden syndrome [63–65]. Currently, it is unclear whether gastric polyps in CS are neoplastic and if CS patients are at increased risk of gastric cancer. Endoscopic upper gastrointestinal tract surveillance is recommended every 2–3 years starting at 15 years of age [12].

Gastric Adenocarcinoma and Proximal Polyposis of the Stomach

Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) is a recently described autosomal dominant syndrome with an unknown genetic cause [70]. Presently, only 6 families have been reported in the literature [70–72].

The following diagnostic criteria have been proposed: (1) gastric polyps restricted to the body and fundus with no evidence of colorectal or duodenal polyposis; (2) >100 polyps carpeting the proximal stomach in the index case or >30 polyps in a first-degree relative of another case; (3) predominantly FGPs, some having regions of dysplasia (or a family member with either dysplastic FGPs or gastric adenocarcinoma); and (4) an autosomal dominant pattern of inheritance. Importantly, patients with other heritable gastric polyposis syndromes and those on acid-suppressive therapy (i.e., sporadic fundic gland polypsis [18]) are excluded. In patients on acid-suppressive therapy, endoscopy should be repeated when the patient is off therapy [70].

Phenotypically, GAPPS is mainly characterized by fundic gland polyposis with areas with dysplasia. Polyps are typically small and carpet the gastric body and fundus. Gastric polyposis can be observed as young as 10 years of age. Occasional hyperplastic, adenomatous, and mixed polyps can be present also. A recent study of one Australian GAPPS family with 15 patients also described hyperproliferative aberrant pits, polypoid foveolar hyperplasia, predominantly gastric and/or hybrid dysplasia [72]. The gastric cancers in GAPPS are typically intestinal-type adenocarcinoma and can occur at a young age (median age 50 years; range 33–75 years) [70, 71].

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1; von Recklinghausen disease) is an autosomal dominant hereditary syndrome caused by germline mutation of the *NF1* gene. Because the *NF1* gene is large, routine genetic testing is impractical. Therefore, NF1 is traditionally defined by clinical criteria (Table 17.2).

The gastrointestinal tract is commonly involved in NF1 (Fig. 17.6) [73]. Gastrointestinal stromal tumors (GIST) represent the most frequent gastrointestinal tract manifestation of NF1 occurring in up to 25 % of patients. NF1 associated GISTs typically lack mutations in *KIT* or *PDGFRA* [74]. Although much less frequent, peripheral nerve sheet tumors of different histological subtypes, including solitary or plexiform neurofibroma and diffuse neurofibromatosis or ganglioneuromatosis, also occur in the gastrointestinal tract of NF1 patients. Lastly, neuroendocrine tumors, particular in the periampullary region, are common in NF1 [73]. Coexistence of GIST and peripheral nerve sheath tumors in the gastrointestinal tract and/or GIST and (peri)ampullary neuroendocrine neoplasm are almost pathognomonic for NF1 [73].

Recently, inflammatory mucosal gastrointestinal polyps have been suggested as another manifestation of NF1 [75]. Although most polyps occur in the colon or small intestines, four of 15 patients had a gastric polyp and two patients had a polyp at the gastroesophageal junction/Z-line. Most patients had between 1 and 3 polyps, but four patients had ten or more (two of these patients had multiple or diffuse polyposis). Unfortunately, genetic testing to exclude other polyposis syndromes was not performed. Histologically these polyps had a variable appearance ranging from juve-

Table 17.2 Diagnostic criteria for neurofibromatosis type 1

Two or more of these criteria are required for diagnosis:
Six or more café au lait macules (>0.5 cm in children or >1.5 cm in adults)
Two or more cutaneous or subcutaneous neurofibromas or one plexiform neurofibroma
Axillary or groin freckling
Optic pathway glioma
Two or more Lisch nodules (iris hamartomas seen on slit lamp examination)
Bony dysplasia (sphenoid wing dysplasia, bowing of long bone +/– pseudoarthrosis)
First-degree relative with NF1

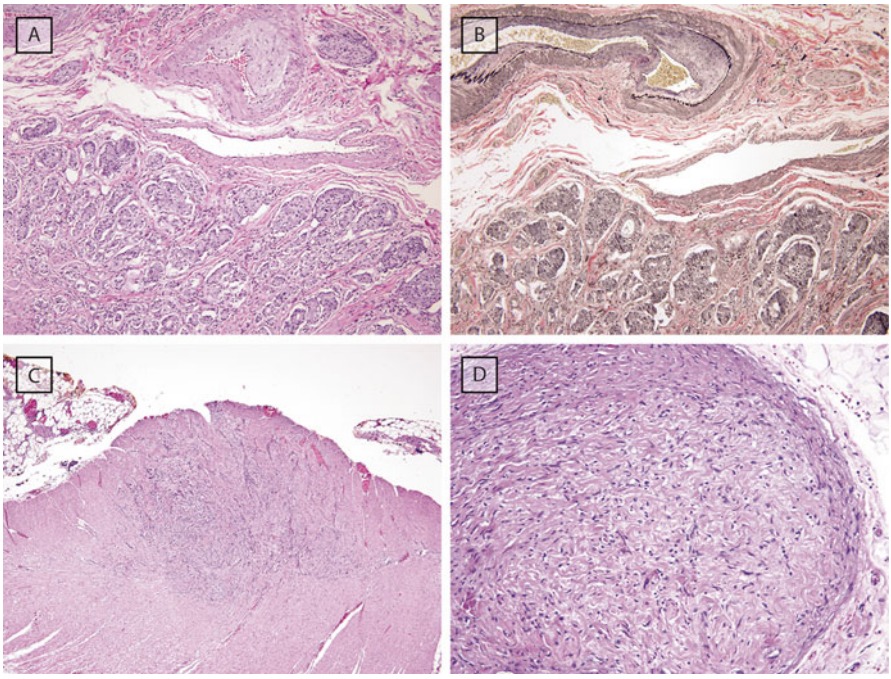

Fig. 17.6 (**a**) Gastrointestinal findings in patients with neurofibromatosis 1. This patient had a somatostatinoma, shown here. This field lacks the characteristic psammoma bodies but shows the vascular intimal hyperplasia accompanying such tumors. (**b**) Gastrointestinal findings in patients with neurofibromatosis 1. This Movat highlights the vascular intimal hyperplasia seen adjacent to the somatostatinoma quite nicely. (**c**) Gastrointestinal findings in patients with neurofibromatosis 1. This field is from the same Whipple operation in which the somatostatinoma in Fig. 17.3a, b was detected. This is a minute gastrointestinal stromal tumor. (**d**) Gastrointestinal findings in patients with neurofibromatosis 1. This field is from the same Whipple operation in which the somatostatinoma in Fig. 17.3a, b was detected. This is a minute neurofibroma

nile-like to granulation tissue rich predominantly inflammatory and hyperplastic. The gastric polyps showed mainly hyperplastic features. There was no dysplasia in any of these polyps which is consistent with the observation that NF1 patients are not at increased risk for colorectal or gastric adenocarcinoma. Based on a pathology database search, the authors estimated the frequency of inflammatory polyps in NF1 to be less than 2 %, the same magnitude as the incidence in the general population [2, 75]. It is therefore questionable whether these polyps are a specific manifestation of NF1 and the true nature and association of these polyps with NF1 remains to be further studied. As an aside, and a source of further confusion, children born with constitutional (biallelic) mismatch repair deficiency can manifest café-au-lait spots seen in neurofibromatosis [76].

McCune–Albright Syndrome

McCune–Albright syndrome (MAS) is a rare sporadic disorder caused by postzygotic activating mutation in the *GNAS* gene. The classical triad of McCune–Albright syndrome consists of polyostotic fibrous dysplasia, skin hyperpigmentation (café-au-lait spots), and endocrine dysfunctions, notably precocious puberty, hyperthyroidism, growth hormone excess, hyperprolactemia, and hypercortisolism. These manifestations usually present during infancy and childhood. Because patients with MAS display mosaicism of activating somatic *GNAS* mutations, the clinical presentation of each individual depends on the particular distribution of affected cells [77].

Recently, upper gastrointestinal polyps were identified as another frequent phenotypic expression of MAS [78]. Duodenal polyps were present in all four patients that were studied and gastric polyps were found in two of these four patients. No colonic polyps were found. Morphologically duodenal polyps showed arborizing smooth muscle fibers in the lamina propria. Gastric polyps showed elongated gastric pits and a mixture of fundic, pyloric, and Brunner's glands. The authors concluded that the polyps in MAS most closely resembled Peutz–Jeghers type hamartomatous polyps, but germline *LKB1* mutations were excluded. Importantly, activating *GNAS* mutations were found in these polyps, confirming the association with MAS molecularly [78]. Another study confirmed the presence of various upper gastrointestinal mass lesions in three patients with MAS [79]. One patient had a circumferential cardiac polypoid gastric adenoma (Fig. 17.7), fundic gland polyps were present in two patients, and a gastric hyperplastic polyp and foveolar hyperplasia were both found in one patient. Multifocal gastric heterotopia in the duodenum was found in two patients. Interestingly, two of these patients received upper endoscopy for evaluation of a pancreatic cyst, likely representing an IPMN, which has been described in MAS before [80]. No high-grade dysplasia was reported and no cases of gastrointestinal cancer in MAS are known. To further define the association between MAS and gastrointestinal polyps, routine endoscopy is recommended for patients with MAS [78, 79].

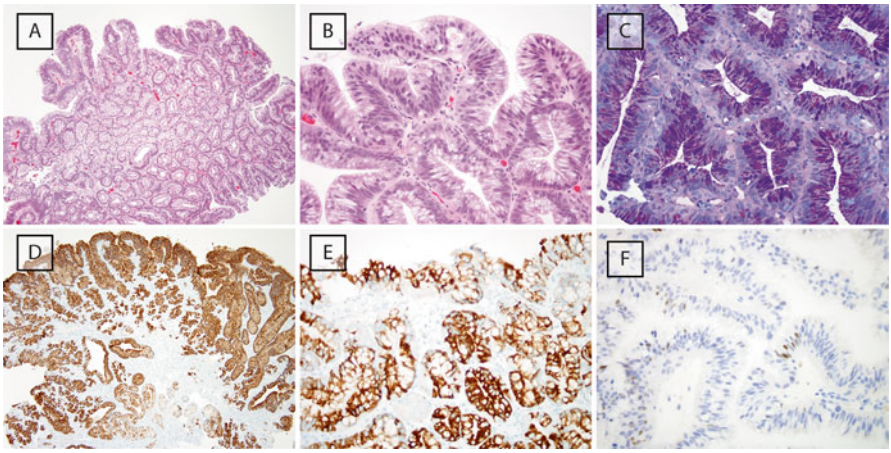

Fig. 17.7 (**a**) Unusual adenoma from a patient with McCune–Albright syndrome. On H&E, the lesion is reminiscent of both foveolar and pyloric-type adenomas but not typical of either. (**b**) Unusual adenoma from a patient with McCune–Albright syndrome. At high magnification there is a suggestion of intestinal differentiation as well as pyloric and foveolar differentiation. (**c**) Unusual adenoma from a patient with McCune–Albright syndrome, PAS/AB stain. Note the presence of goblet cells as well as neutral mucin and the ground glass appearance that characterizes pyloric type differentiation. (**d**) Unusual adenoma from a patient with McCune–Albright syndrome, MUC5AC stain. This confirms gastric foveolar differentiation. (**e**) Unusual adenoma from a patient with McCune–Albright syndrome, MUC6 stain. This confirms pyloric gland differentiation. (**f**) Unusual adenoma from a patient with McCune–Albright syndrome, CDX2 stain. This confirms intestinal differentiation

Cronkhite–Canada Syndrome

Cronkhite–Canada syndrome (CCS) is a rare protein-losing enteropathy typically characterized by diffuse gastrointestinal polyposis and typical ectodermal changes, such as hair loss and nail dystrophy. Although recent studies favor an autoimmune etiology, the precise cause of CCS has not been elucidated [81]. More than 80 % of patients are diagnosed at age 50 or older. The prognosis is poor. Less than 5 % of patients have complete remission and a 5-year mortality rate of 55 % is noted due to gastrointestinal bleeding, sepsis, and congestive heart failure. There is no standard therapy but limited success has been reported with antibiotics, steroids, and partial gastrectomy [81, 82].

Typically polyposis in CCS is diffuse throughout the entire gastrointestinal tract. The esophagus is uninvolved. The polyps are broad based and sessile and are a few millimeters to 1.5 cm in size. In the upper gastrointestinal tract, diffuse mucosal thickening rather than polyposis can be the main endoscopic picture (Fig. 17.8), which may be more suggestive for gastric malignancy (lymphoma of linitis plastica) or gastric infection than CCS polyposis [83].

Microscopically polyps in CCS show marked foveolar hyperplasia with cystically dilated glands, abundant stromal edema, and a predominantly mononuclear inflammatory infiltrate (Fig. 17.8). Eosinophils can be prominent. Especially in the stomach, the

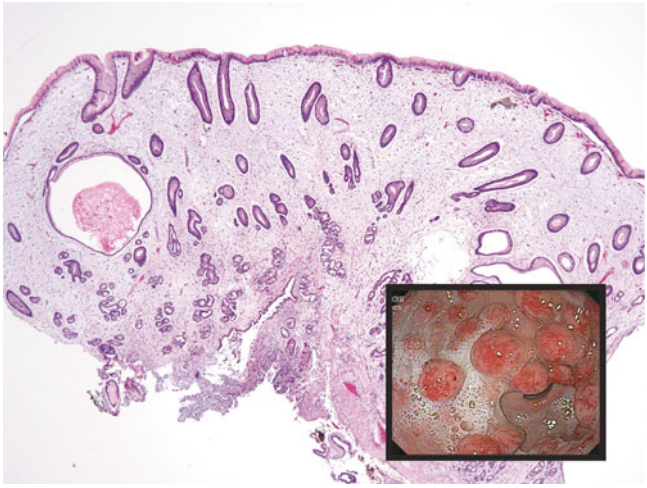

Fig. 17.8 Gastric Cronkhite–Canada polyposis. The polyps appear very similar to juvenile and hyperplastic polyps. The main clue is that flat mucosa is abnormal and the patients are very ill from profuse protein loss. Inset: endoscopic appearance of gastric Cronkhite–Canada polyposis. Numerous polyps are encountered throughout the gastrointestinal tract, sparing the esophagus. The flat mucosa is also abnormal

polyps can be difficult to discriminate from hyperplastic, juvenile or Peutz–Jeghers polyps [84]. Of key diagnostic importance for this differential diagnosis is that the intervening endoscopically nonaffected mucosa in CCS is also affected and shows marked lamina propria edema, inflammatory infiltrate, and gland distortion, which is not seen in JPS or PJS [81, 84]. In addition, correlation with clinical manifestations, in particular the typical ectodermal changes in CCS, is key to a correct diagnosis [84]. Ménétrier's disease is another important differential diagnosis, both clinically as well as histologically. However, the hyperplastic pathology in Menetrier disease is normally limited to the foveolar compartment of the body and fundus with unremarkable antral mucosa and without lamina propria edema [81].

Polyps in CCS are nonneoplastic but coexisting adenomas and adenocarcinomas have been reported. Patients may be at increased risk of colorectal cancer, possibly secondary to chronic mucosal inflammation. However, assessment of gastrointestinal cancer risk is limited by the rarity of this syndrome, and it remains inconclusive whether CCS patients are truly at increased risk of gastrointestinal malignancy [81].

Conclusion

Gastric polyps are found in 1–4 % of gastroscopic procedures. Most of these polyps are sporadic lesions, but polyps in the stomach can also indicate an underlying syndrome (Table 17.1). Polyps in a syndromic setting are often multiple, as in FAP, JPS, or PJS, but can also be few in number such as in Lynch syndrome. Different

histologic types of polyps can be found in different syndromes, which can help establish a syndromic diagnosis. However, the histology of gastric polyps is less specific compared to colonic polyps, and one should be careful to establish a diagnosis of a polyposis syndrome solely based on the gastric polyp pathology [10, 83, 84].

Some of the syndromes described in this chapter are associated with an increased risk of gastric cancer (e.g., LS, PJS, and JPS), whereas others are not (e.g., FAP, MAP, MAS). Interestingly, the assumption that the polyp is the precursor lesion of gastric cancer in these syndromes is not always true. For instance, whereas proof exists that juvenile polyps can be precursor lesions of gastric cancer in JPS, this is not the case in PJS. Moreover, Western FAP patients typically have numerous gastric polyps, mostly fundic gland polyps and some gastric foveolar adenomas, but do not have an increased risk of gastric cancer [27].

Prevention of upper gastrointestinal cancer is one of the major reasons for endoscopic surveillance of patients with syndromic polyps. Well-defined precursor lesions of gastric cancer that can be screened for and treated are a sine qua non for successful endoscopic surveillance. In syndromes where polyps are the (only) precursor lesions of gastric cancer it is likely effective to remove these polyps to prevent cancer development. However, screening for gastric cancer is more difficult in syndromes with an increased risk of gastric cancer but without a well-defined precursor lesion (such as LS or PJS) and other biomarkers are needed. Studying tumorigenesis in these syndromes will increase understanding of gastric cancer carcinogenesis and may identify new biomarkers or precursor lesions leading to more effective screening methods. As such, gastric polyric gland adenomas were recently suggested to be precursor lesions of gastric cancer in Lynch syndrome [9].

Moreover, the neoplastic potential of Peutz–Jeghers polyps is a matter of debate. Dysplasia in a Peutz–Jeghers polyp is very rare and it has been suggested that Peutz–Jeghers polyps are in fact an epiphenomenon to the cancer prone condition and not obligate malignant precursors [44]. Indeed, a protracted clonal evolution has been shown in normal colonic crypts from PJS patients. This allows a greater number of mutations to be retained in the crypt which accelerates somatic evolution and can explain the increased risk of colorectal cancer in PJS [45]. These alterations in stem cell dynamics in morphologically nonneoplastic mucosa may ultimately be used as a biomarker for cancer risk. Pretumor progression has not yet been studied in gastric mucosa.

To conclude, studying genetic syndromes that lead to disturbed epithelial homeostasis, polyp formation, and sometimes gastric cancer can greatly increase our understanding of gastric tumorigenesis. This will improve patient care and can lead to new biomarkers that can be used in screening of patients at risk for gastric cancer.

References

1. Carmack SW, Genta RM, Graham DY, et al. Management of gastric polyps: a pathology-based guide for gastroenterologists. Nat Rev Gastroenterol Hepatol. 2009;6:331–41.
2. Carmack SW, Genta RM, Schuler CM, et al. The current spectrum of gastric polyps: a 1-year national study of over 120,000 patients. Am J Gastroenterol. 2009;104:1524–32.

3. Gonzalez-Obeso E, Fujita H, Deshpande V, et al. Gastric hyperplastic polyps: a heterogeneous clinicopathologic group including a distinct subset best categorized as mucosal prolapse polyp. Am J Surg Pathol. 2011;35:670–7.
4. Abraham SC, Singh VK, Yardley JH, et al. Hyperplastic polyps of the stomach: associations with histologic patterns of gastritis and gastric atrophy. Am J Surg Pathol. 2001;25:500–7.
5. Abraham SC, Montgomery EA, Singh VK, et al. Gastric adenomas: intestinal-type and gastric-type adenomas differ in the risk of adenocarcinoma and presence of background mucosal pathology. Am J Surg Pathol. 2002;26:1276–85.
6. Vieth M, Montgomery EA. Some observations on pyloric gland adenoma: an uncommon and long ignored entity! J Clin Pathol. 2014;67:883–90.
7. Matsubara A, Sekine S, Kushima R, et al. Frequent GNAS and KRAS mutations in pyloric gland adenoma of the stomach and duodenum. J Pathol. 2013;229:579–87.
8. Wood LD, Salaria SN, Cruise MW, et al. Upper GI tract lesions in familial adenomatous polyposis (FAP): enrichment of pyloric gland adenomas and other gastric and duodenal neoplasms. Am J Surg Pathol. 2014;38:389–93.
9. Lee SE, Kang SY, Cho J, et al. Pyloric gland adenoma in Lynch syndrome. Am J Surg Pathol. 2014;38:784–92.
10. Lam-Himlin D, Park JY, Cornish TC, et al. Morphologic characterization of syndromic gastric polyps. Am J Surg Pathol. 2010;34:1656–62.
11. Jansen M, Menko FH, Brosens LA, et al. Establishing a clinical and molecular diagnosis for hereditary colorectal cancer syndromes: present tense, future perfect? Gastrointest Endosc. 2014;80:1145–55.
12. Syngal S, Brand RE, Church JM, et al. ACG clinical guideline: genetic testing and management of hereditary gastrointestinal cancer syndromes. Am J Gastroenterol. 2015;110:223–62.
13. Nielsen M, Hes FJ, Nagengast FM, et al. Germline mutations in APC and MUTYH are responsible for the majority of families with attenuated familial adenomatous polyposis. Clin Genet. 2007;71:427–33.
14. Nielsen M, Morreau H, Vasen HF, et al. MUTYH-associated polyposis (MAP). Crit Rev Oncol Hematol. 2011;79:1–16.
15. Brosens LA, Keller JJ, Offerhaus GJ, et al. Prevention and management of duodenal polyps in familial adenomatous polyposis. Gut. 2005;54:1034–43.
16. Bianchi LK, Burke CA, Bennett AE, et al. Fundic gland polyp dysplasia is common in familial adenomatous polyposis. Clin Gastroenterol Hepatol. 2008;6:180–5.
17. Attard TM, Cuffari C, Tajouri T, et al. Multicenter experience with upper gastrointestinal polyps in pediatric patients with familial adenomatous polyposis. Am J Gastroenterol. 2004;99:681–6.
18. Torbenson M, Lee JH, Cruz-Correa M, et al. Sporadic fundic gland polyposis: a clinical, histological, and molecular analysis. Mod Pathol. 2002;15:718–23.
19. Abraham SC, Nobukawa B, Giardiello FM, et al. Sporadic fundic gland polyps: common gastric polyps arising through activating mutations in the beta-catenin gene. Am J Pathol. 2001;158:1005–10.
20. Genta RM, Schuler CM, Robiou CI, et al. No association between gastric fundic gland polyps and gastrointestinal neoplasia in a study of over 100,000 patients. Clin Gastroenterol Hepatol. 2009;7:849–54.
21. Zwick A, Munir M, Ryan CK, et al. Gastric adenocarcinoma and dysplasia in fundic gland polyps of a patient with attenuated adenomatous polyposis coli. Gastroenterology. 1997;113:659–63.
22. Arnason T, Liang WY, Alfaro E, et al. Morphology and natural history of familial adenomatous polyposis-associated dysplastic fundic gland polyps. Histopathology. 2014;65:353–62.
23. Abraham SC, Nobukawa B, Giardiello FM, et al. Fundic gland polyps in familial adenomatous polyposis: neoplasms with frequent somatic adenomatous polyposis coli gene alterations. Am J Pathol. 2000;157:747–54.
24. Abraham SC, Park SJ, Mugartegui L, et al. Sporadic fundic gland polyps with epithelial dysplasia: evidence for preferential targeting for mutations in the adenomatous polyposis coli gene. Am J Pathol. 2002;161:1735–42.

25. Park JY, Cornish TC, Lam-Himlin D, et al. Gastric lesions in patients with autoimmune metaplastic atrophic gastritis (AMAG) in a tertiary care setting. Am J Surg Pathol. 2010;34:1591–8.
26. Nakamura S, Matsumoto T, Kobori Y, et al. Impact of helicobacter pylori infection and mucosal atrophy on gastric lesions in patients with familial adenomatous polyposis. Gut. 2002;51:485–9.
27. Offerhaus GJ, Giardiello FM, Krush AJ, et al. The risk of upper gastrointestinal cancer in familial adenomatous polyposis. Gastroenterology. 1992;102:1980–2.
28. Park JG, Park KJ, Ahn YO, et al. Risk of gastric cancer among Korean familial adenomatous polyposis patients. Report of three cases. Dis Colon Rectum. 1992;35:996–8.
29. Iwama T, Mishima Y, Utsunomiya J. The impact of familial adenomatous polyposis on the tumorigenesis and mortality at the several organs. Its rational treatment. Ann Surg. 1993;217:101–8.
30. Leggett B. FAP: another indication to treat H pylori. Gut. 2002;51:463–4.
31. Boparai KS, Dekker E, Van Eeden S, et al. Hyperplastic polyps and sessile serrated adenomas as a phenotypic expression of MYH-associated polyposis. Gastroenterology. 2008;135:2014–8.
32. Vogt S, Jones N, Christian D, et al. Expanded extracolonic tumor spectrum in MUTYH-associated polyposis. Gastroenterology. 2009;137:1976–85 e1-10.
33. Wiland H, Kalady H, Heald B, et al. Clinical and histopathologic characterization of polyp burden in patients with MUTYH-associated polyposis. Mod Pathol. 2015;28:198A.
34. Peltomäki PT, Offerhaus GJ, Vasen HFA. Lynch syndrome. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. WHO classification of tumours of the digestive system. Lyon: IARC Press; 2010.
35. Aarnio M, Salovaara R, Aaltonen LA, et al. Features of gastric cancer in hereditary non-polyposis colorectal cancer syndrome. Int J Cancer. 1997;74:551–5.
36. Capelle LG, Van Grieken NC, Lingsma HF, et al. Risk and epidemiological time trends of gastric cancer in Lynch syndrome carriers in the Netherlands. Gastroenterology. 2010;138:487–92.
37. Edelstein DL, Axilbund J, Baxter M, et al. Rapid development of colorectal neoplasia in patients with Lynch syndrome. Clin Gastroenterol Hepatol. 2011;9:340–3.
38. Renkonen-Sinisalo L, Sipponen P, Aarnio M, et al. No support for endoscopic surveillance for gastric cancer in hereditary non-polyposis colorectal cancer. Scand J Gastroenterol. 2002;37:574–7.
39. Brosens LA, van Hattem WA, Jansen M, et al. Gastrointestinal polyposis syndromes. Curr Mol Med. 2007;7:29–46.
40. McGarrity TJ, Kulin HE, Zaino RJ. Peutz-Jeghers syndrome. Am J Gastroenterol. 2000;95:596–604.
41. Tse JY, Wu S, Shinagare SA, et al. Peutz-Jeghers syndrome: a critical look at colonic Peutz-Jeghers polyps. Mod Pathol. 2013;26:1235–40.
42. van Lier MG, Westerman AM, Wagner A, et al. High cancer risk and increased mortality in patients with Peutz-Jeghers syndrome. Gut. 2011;60:141–7.
43. Giardiello FM, Brensinger JD, Tersmette AC, et al. Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology. 2000;119:1447–53.
44. Jansen M, de Leng WW, Baas AF, et al. Mucosal prolapse in the pathogenesis of Peutz-Jeghers polyposis. Gut. 2006;55:1–5.
45. Langeveld D, Jansen M, de Boer DV, et al. Aberrant intestinal stem cell lineage dynamics in Peutz-Jeghers syndrome and familial adenomatous polyposis consistent with protracted clonal evolution in the crypt. Gut. 2012;61:839–46.
46. Brosens LA, Langeveld D, van Hattem WA, et al. Juvenile polyposis syndrome. World J Gastroenterol. 2011;17:4839–44.
47. van Hattem WA, Brosens LA, de Leng WW, et al. Large genomic deletions of SMAD4, BMPR1A and PTEN in juvenile polyposis. Gut. 2008;57:623–7.
48. Delnatte C, Sanlaville D, Mougenot JF, et al. Contiguous gene deletion within chromosome arm 10q is associated with juvenile polyposis of infancy, reflecting cooperation between the BMPR1A and PTEN tumor-suppressor genes. Am J Hum Genet. 2006;78:1066–74.

49. Menko FH, Kneepkens CM, de Leeuw N, et al. Variable phenotypes associated with 10q23 microdeletions involving the PTEN and BMPR1A genes. Clin Genet. 2008;74:145–54.
50. Salviati L, Patricelli M, Guariso G, et al. Deletion of PTEN and BMPR1A on chromosome 10q23 is not always associated with juvenile polyposis of infancy. Am J Hum Genet. 2006;79:593–6. author reply 596–7.
51. Brosens LA, van Hattem A, Hylind LM, et al. Risk of colorectal cancer in juvenile polyposis. Gut. 2007;56:965–7.
52. Howe JR, Mitros FA, Summers RW. The risk of gastrointestinal carcinoma in familial juvenile polyposis. Ann Surg Oncol. 1998;5:751–6.
53. Friedl W, Uhlhaas S, Schulmann K, et al. Juvenile polyposis: massive gastric polyposis is more common in MADH4 mutation carriers than in BMPR1A mutation carriers. Hum Genet. 2002;111:108–11.
54. Aretz S, Stienen D, Uhlhaas S, et al. High proportion of large genomic deletions and a genotype phenotype update in 80 unrelated families with juvenile polyposis syndrome. J Med Genet. 2007;44:702–9.
55. Jarvinen HJ, Sipponen P. Gastroduodenal polyps in familial adenomatous and juvenile polyposis. Endoscopy. 1986;18:230–4.
56. Ma C, Giardiello FM, Montgomery EA. Upper tract juvenile polyps in juvenile polyposis patients: dysplasia and malignancy are associated with foveolar, intestinal, and pyloric differentiation. Am J Surg Pathol. 2014;38:1618–26.
57. Postgate AJ, Will OC, Fraser CH, et al. Capsule endoscopy for the small bowel in juvenile polyposis syndrome: a case series. Endoscopy. 2009;41:1001–4.
58. Woodford-Richens K, Bevan S, Churchman M, et al. Analysis of genetic and phenotypic heterogeneity in juvenile polyposis. Gut. 2000;46:656–60.
59. Sayed MG, Ahmed AF, Ringold JR, et al. Germline SMAD4 or BMPR1A mutations and phenotype of juvenile polyposis. Ann Surg Oncol. 2002;9:901–6.
60. Handra-Luca A, Condroyer C, de Moncuit C, et al. Vessels' morphology in SMAD4 and BMPR1A-related juvenile polyposis. Am J Med Genet A. 2005;138:113–7.
61. Hizawa K, Iida M, Yao T, et al. Juvenile polyposis of the stomach: clinicopathological features and its malignant potential. J Clin Pathol. 1997;50:771–4.
62. Langeveld D, van Hattem WA, de Leng WW, et al. SMAD4 immunohistochemistry reflects genetic status in juvenile polyposis syndrome. Clin Cancer Res. 2010;16:4126–34.
63. Pilarski R, Burt R, Kohlman W, et al. Cowden syndrome and the PTEN hamartoma tumor syndrome: systematic review and revised diagnostic criteria. J Natl Cancer Inst. 2013;105:1607–16.
64. Heald B, Mester J, Rybicki L, et al. Frequent gastrointestinal polyps and colorectal adenocarcinomas in a prospective series of PTEN mutation carriers. Gastroenterology. 2010;139:1927–33.
65. Borowsky J, Setia N, Lauwers G, et al. Gastrointestinal tract pathology in PTEN hamartoma tumour syndrome: a review of 43 cases. Mod Pathol. 2015;28:149A.
66. Coriat R, Mozer M, Caux F, et al. Endoscopic findings in Cowden syndrome. Endoscopy. 2011;43:723–6.
67. Levi Z, Baris HN, Kedar I, et al. Upper and lower gastrointestinal findings in PTEN mutation-positive Cowden syndrome patients participating in an active surveillance program. Clin Transl Gastroenterol. 2011;2, e5.
68. Al-Thihli K, Palma L, Marcus V, et al. A case of Cowden's syndrome presenting with gastric carcinomas and gastrointestinal polyposis. Nat Clin Pract Gastroenterol Hepatol. 2009;6:184–9.
69. Hamby LS, Lee EY, Schwartz RW. Parathyroid adenoma and gastric carcinoma as manifestations of Cowden's disease. Surgery. 1995;118:115–7.
70. Worthley DL, Phillips KD, Wayte N, et al. Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): a new autosomal dominant syndrome. Gut. 2012;61:774–9.
71. Yanaru-Fujisawa R, Nakamura S, Moriyama T, et al. Familial fundic gland polyposis with gastric cancer. Gut. 2012;61:1103–4.

72. de Boer WB, Kumarasinghe M, Ee H. Gastric adenocarcinoma and proximal polyposis syndrome (GAPPS): not just fundic gland polyps. Mod Pathol. 2015;28:156A.
73. Agaimy A, Vassos N, Croner RS. Gastrointestinal manifestations of neurofibromatosis type 1 (Recklinghausen's disease): clinicopathological spectrum with pathogenetic considerations. Int J Clin Exp Pathol. 2012;5:852–62.
74. Miettinen M, Fetsch JF, Sobin LH, et al. Gastrointestinal stromal tumors in patients with neurofibromatosis 1: a clinicopathologic and molecular genetic study of 45 cases. Am J Surg Pathol. 2006;30:90–6.
75. Agaimy A, Schaefer IM, Kotzina L, et al. Juvenile-like (inflammatory/hyperplastic) mucosal polyps of the gastrointestinal tract in neurofibromatosis type 1. Histopathology. 2014;64: 777–86.
76. Durno CA, Holter S, Sherman PM, et al. The gastrointestinal phenotype of germline biallelic mismatch repair gene mutations. Am J Gastroenterol. 2010;105:2449–56.
77. Volkl TM, Dorr HG. McCune-Albright syndrome: clinical picture and natural history in children and adolescents. J Pediatr Endocrinol Metab. 2006;19 Suppl 2:551–9.
78. Zacharin M, Bajpai A, Chow CW, et al. Gastrointestinal polyps in McCune Albright syndrome. J Med Genet. 2011;48:458–61.
79. Bhaijee F, Pittman M, Robertson S, et al. Upper gastrointestinal polyps in McCune Albright syndrome. Mod Pathol. 2015;28:148A.
80. Parvanescu A, Cros J, Ronot M, et al. Lessons from McCune-Albright syndrome-associated intraductal papillary mucinous neoplasms: GNAS-activating mutations in pancreatic carcinogenesis. JAMA Surg. 2014;149:858–62.
81. Slavik T, Montgomery EA. Cronkhite-Canada syndrome six decades on: the many faces of an enigmatic disease. J Clin Pathol. 2014;67:891–7.
82. Daniel ES, Ludwig SL, Lewin KJ, et al. The Cronkhite-Canada syndrome. An analysis of clinical and pathologic features and therapy in 55 patients. Medicine. 1982;61:293–309.
83. Bettington M, Brown IS, Kumarasinghe MP, et al. The challenging diagnosis of Cronkhite-Canada syndrome in the upper gastrointestinal tract: a series of 7 cases with clinical follow-up. Am J Surg Pathol. 2014;38:215–23.
84. Burke AP, Sobin LH. The pathology of Cronkhite-Canada polyps. A comparison to juvenile polyposis. Am J Surg Pathol. 1989;13:940–6.

Chapter 18
Histopathological, Molecular, and Genetic Profile of Hereditary Diffuse Gastric Cancer: Current Knowledge and Challenges for the Future

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Introduction

Gastric cancer (GC) is the fifth leading cause of cancer globally and ranks third in terms of cancer-related mortality [1]. GCs display various morphological phenotypes reflected in a large number of suggested histopathological classification schemes. The most commonly used are the classification of the World Health Organization (WHO) [2] and the classification by Laurén [3]. The Laurén classification is often used to classify GC into three broad categories, namely intestinal type, diffuse type, and a remaining group of GC that cannot be placed in one of these two categories [3]. Intestinal type GC is composed of tumor cells with glandular,

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tubular, or papillary growth pattern with various degrees of differentiation. Diffuse type GC consists of solitary or small clusters of poorly cohesive cells that frequently infiltrate in a diffuse pattern with or without a small component of gland formation. Typical signet ring cells (SRCs) often characterize diffuse GC and when the tumor is composed of predominantly (more than 50 %) SRCs, the tumor is also referred to as signet ring cell carcinoma (SRCC).

Although most GCs are sporadic, familial aggregation is known to occur in around 10–20 % of patients. Incidences described range from 2.8 % in Sweden to 36.6 % in Japan and are different between low- and high-risk areas [4–7]. Familial gastric cancer can be classified as hereditary diffuse gastric cancer (HDGC), familial intestinal gastric cancer (FIGC) and, when the histopathology of tumors is unknown, as familial gastric cancer (FGC) [8]. Among these groups, only 1–3 % are related to known specific genetic causes with the most important GC susceptibility gene for HDGC being *CDH1*.

In 2012, a new hereditary gastric cancer syndrome was identified, and was coined GAPPS (Gastric Adenocarcinoma and Proximal Polyposis of the Stomach), which is an autosomal dominant condition characterized by fundic gland polyposis with increased risk of developing intestinal type GC and so far unknown genetic cause [9, 10].

Moreover, GC risk is elevated in several other hereditary cancer syndromes, namely Lynch syndrome caused by germline mutations in one of the DNA mismatch repair genes [11–13], Li-Fraumeni syndrome caused by *TP53* germline mutations [14–16], familial adenomatous polyposis caused by *APC* germline mutations [17, 18],

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Peutz-Jeghers syndrome caused by *STK11* germline mutations [19–21], juvenile polyposis syndrome caused by *SMAD4* or *BMPR1A* germline mutations [22, 23], and hereditary breast or ovarian cancer syndrome caused by *BRCA1* or *BRCA2* germline mutations [10, 24, 25].

In this chapter, we discuss the current knowledge of HDGC, particularly *CDH1* mutation-related HDGC, and provide new insights into the phenotypic characteristics of early and advanced HDGCs using immunohistochemical biomarkers of cell adhesion, proliferation, anoikis, epithelial-mesenchymal-transition, and cancer cell stemness.

Genetics of HDGC

Already in 1964, Jones reported familial clustering of GC among a large Māori kindred in New Zealand [26]. However, it took until 1998 to identify germline mutations in *CDH1* in three Maori families as the cause of HDGC by linkage analysis [27]. The E-cadherin gene, *CDH1,* is located on chromosome 16q22.1. The 120 kDa glycoprotein encoded by *CDH1* displays a large extracellular domain, a transmembrane segment and a short cytoplasmic domain [28]. E-cadherin is a transmembrane calcium-dependent protein and is mainly expressed at the basolateral membrane of epithelial cells, where it has important roles in cell-cell adhesion at the *adherens* junctions to maintain epithelial integrity [29, 30]. Heterozygous germline *CDH1* mutations have been described in 18–40 % of HDGC families [31–35]. The frequency of *CDH1* mutations seems to be highly variable, which may be related to the variable incidence of GC across different geographic regions. Overall, in more than 60 % of HDGC families, the role of *CDH1* germline deficiency is unclear.

There are a few other genes which are involved in HDGC predisposition, including *CTNNA1*. Like *CDH1*, *CTNNA1* is involved in intercellular cell adhesion. *CTNNA1* encodes the protein α-E-catenin, which functions in a complex with β-catenin where it binds the cytoplasmic domain of E-cadherin to the cytoskeleton [36, 37]. α-E-catenin inhibition has been shown to destabilize adherens junctions, weakening the interaction between cells [38]. Currently, three families have been described with *CTNNA1* germline mutations [35, 39]. Loss of α-E-catenin expression with preservation of E-cadherin has been observed in GC identified in *CTNNA1* mutation carriers. These families show a clinical picture similar to that of *CDH1*-mutation positive families, however there is insufficient data available to make a statement on disease penetrance.

There are HDGC families with mutations in genes associated with other cancer-predisposition syndromes, such as *BRCA2*. Germline *BRCA2* mutations predispose to hereditary breast and ovarian cancer. In some families with *BRCA2* mutations, an increased incidence of GC has been encountered [40–43], with one family fulfilling the HDGC criteria [35].

Germline mutations in *MAP3K6 gene* have been described in families with FGC, although the role of these mutations is yet to be proven [44]. In *CDH1*-negative HDGC families, multiplexed targeted sequencing of cancer associated genes led recently to the identification of new germline mutations in several genes, such as

CTNNA1, *BRCA2*, *STK11*, *SDHB*, *PRSS1*, *ATM*, *MSR1*, and *PALB2* [35]. It is likely that other HDGC associated genes will be discovered in the near future through next-generation-sequencing (NGS) empowered methodologies. However, to assess pathogenicity, disease penetrance and management for newly identified gene mutations, multiple mutation positive families have to be studied and outcomes have to be collected at a global level.

Germline CDH1 *Mutation and Clinical Guidelines*

HDGC caused by germline *CDH1* mutations is an autosomal dominant cancer-susceptibility syndrome. Germline *CDH1* alterations can affect the entire coding sequence including small frameshifts, splice-site, nonsense, missense mutations as well as large rearrangements [45, 46]. Most truncating mutations in *CDH1* are pathogenic and several missense *CDH1* mutations have been shown to have a deleterious effect on E-cadherin function [10].

Mutation carriers have an increased risk of developing diffuse type GC (DGC) as well as lobular breast cancer (LBC). In a recent study, penetrance data for *CDH1* mutation carriers has been updated based on affected individuals, who presented clinically with DGC or LBC, from 75 families with germline, pathogenic truncating *CDH1* mutations [35]. The cumulative risk of DGC for *CDH1* mutation carriers by the age of 80 years is reported to be 70 % for men (95 % confidence interval [95 % CI], 59–80 %) and 56 % for women (95 % CI, 44–69 %), though there is no clear explanation why this risk is different for men and women. Furthermore, the cumulative risk of LBC for women with a *CDH1* mutation by the age of 80 years is estimated to be 42 % (95 % CI, 23–68 %) [35]. There is currently no evidence that the risk of other cancer types in individuals with a *CDH1* mutation is significantly increased.

In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) defined families with the HDGC syndrome (OMIM #137215) as those fulfilling one of the two following criteria [8]:

1. Two or more documented cases of diffuse gastric cancer (DGC) in first- or second-degree relatives, with at least one being diagnosed before the age of 50 years; or
2. Three or more cases of DGC in first- or second-degree relatives, independent of age of diagnosis.

Families with aggregation of GC and an index case with DGC, but not fulfilling the IGCLC criteria for HDGC, should be classified as familial diffuse gastric cancer (FDGC) [8]. IGCLC criteria were updated in 2010 [45] and again more recently in 2015 [47]. The recently published guideline broadened the clinical criteria to select patients for *CDH1* mutation analysis. The above mentioned criteria were merged into a new criterion: “Two or more GC cases regardless of age, at least one confirmed DGC, in first- and second-degree relatives.” In addition, new criteria were

Table 18.1 Criteria for *CDH1* testing, according to the updated IGCLC guideline [47]

CDH1 testing criteria[a]
Two or more GC cases, regardless of age, at least one confirmed DGC
One case of DGC <40 years
Personal or family history of DGC and LBC, one diagnosis <50 years
Families in whom testing should be considered[a]
Bilateral LBC or family history of two or more LBC cases <50 years
Personal or family history of cleft lip/palate and DGC
In situ signet ring cells and/or pagetoid spread of signet ring cells

[a]Including both first- and second-degree relatives

added for genetic testing and family management, including families with: bilateral or multiple cases of LBC without DGC, and families with DGC and cleft lip/cleft palate. The criteria are described in Table 18.1.

CDH1 germline mutation testing should be performed in probands affected by either DGC or LBC. Screening of at-risk individuals is indicated from the age of consent, after counseling in a multidisciplinary team. Since *CDH1* mutation carriers have a considerable increased risk to develop invasive GC, which is associated with high mortality, prophylactic total gastrectomy is advised for individuals with a proven pathogenic germline *CDH1* mutation, as it is the only option to eliminate the risk of DGC development [45, 47]. In the prophylactic gastrectomy specimens of these individuals, multiple SRCCs can usually be found. In individuals with proven pathogenic *CDH1* mutations who decline to undergo prophylactic gastrectomy, endoscopic surveillance with multiple biopsies according to the Cambridge protocol is advised [47]. Endoscopic screening in a research setting is also recommended for patients with a *CDH1* variant of unknown significance, or with HDGC without a proven *CDH1* mutation. In one case series, intramucosal SRCCs were detected endoscopically in 2 of 7 *CDH1* mutation-negative individuals (1/5 families) [48].

Somatic Changes in HDGC

Inactivation of the 2nd CDH1 *Allele*

Individuals with a germline *CDH1* mutation, have a single functional *CDH1* allele. When this wild-type allele becomes inactivated by a somatic second-hit molecular mechanism, this leads to biallelic inactivation of the *CDH1* gene and the development of DGC [49–51]. Initial reports indicated that the second-hit that inactivates

CDH1 in HDGC is most commonly promoter hypermethylation [49, 51]. In 2009, Oliveira et al. performed a systematic study to establish the frequency of different types of somatic *CDH1* second-hits occurring in *CDH1*-related GC [52]. This study confirmed that promoter hypermethylation was the most frequent second *CDH1* hit, identified in 32.1 % of the lesions analyzed, whereas loss of heterozygosity was found in 25 %, both alterations in 17.9 % and no alterations in 25 %, when both primary GC and lymph node metastases were analyzed [52]. In fact, 50 % of primary GC displayed *CDH1* epigenetic modifications as a second-hit, whereas in GC metastases the most common second-hit was loss of heterozygosity. Different neoplastic lesions from the same patient frequently displayed different types of second-hits and different types of second-hits were also found within the same tumor sample [49, 51, 52]. These results demonstrated substantial heterogeneity in the mechanisms that can act as *CDH1* second-hits in a single patient.

Other Somatic Changes in HDGC

There has been no systematic study of somatic genetic and epigenetic alterations in genes other than *CDH1* in HDGC from *CDH1* germline mutation carriers. Thus, at this moment in time, there remains a lack of understanding of the cascade of genetic or epigenetic events taking place after *CDH1* inactivation by a second-hit. Such knowledge is necessary to shed light onto the players involved in the evolution from early to invasive HDGC lesions.

Exome sequencing of a single HDGC has been performed. However, in this case, the underlying family predisposing gene was *CTNNA1* and not *CDH1* [39], and somatic mutations at *LMTK3*, *MCTP2*, *MED12*, *PIK3CA*, and *ARID1A* genes have been demonstrated, as well as mutations in other genes recently shown to be part of the molecular signatures of sporadic GC [53–58]. Similar studies in a series of HDGC caused either by *CDH1* or *CTNNA1* germline mutations, and in different progression stages, would undoubtedly help to disclose the somatic mutation landscape of this disease.

Histopathology of HDGC

Prophylactic Gastrectomy

The gross appearances of stomachs from asymptomatic *CDH1* mutation carriers that undergo prophylactic gastrectomy and index patients that present with widely invasive GC differ greatly. The prophylactic gastrectomy specimens generally show no macroscopic abnormalities [59–61]. Sometimes, subtle “pale” areas are visible endoscopically which may represent small foci of SRCs [47, 62]. Macroscopic

examination and sampling of prophylactic gastrectomies should follow specific protocols. Pathological analysis of the entire gastrectomy specimen includes a thorough microscopic assessment using Haematoxylin and Eosin (H&E) and Periodic Acid-Schiff-Diastase (PAS-D) stain.

Microscopically, there are almost always multiple, ranging from a few to dozens, intramucosal cancer foci (pT1a) identified in prophylactic gastrectomy specimens if these are completely processed into paraffin blocks. These tiny (<0.1–10 mm) foci are restricted to the superficial lamina propria and composed of SRCs that are relatively small at the neck-zone level and usually enlarge towards the surface of the gastric mucosa [63]. Considering different studies which reported systematic complete mapping of total gastrectomies, there seems to be no restriction or convincingly preferred location of intramucosal SRCC in the stomach [59–61, 63–67]. The foci were identified from cardia to pre-pyloric region and even in gastric metaplasia beyond the pylorus [47]. As all regions can be affected, pathological examination of the resected specimen should include confirmation of the presence of a complete cuff of proximal squamous oesophageal mucosa and distal duodenal mucosa.

Two typical features of intraepithelial SRCC, which are considered as precursors and only described in *CDH1* mutation carriers, include:

- In situ SRCC (Tis), which corresponds to the substitution of normal epithelial cells of a gland or foveolae by disorganized SRCs that remain within the basement membrane. These tumor cells have hyperchromatic and depolarized nuclei.
- Pagetoid spread of SRCs (Tis), which corresponds to a row of SRCs between the normal epithelial cells and the still intact basal membrane.

In situ SRCC and pagetoid spread of SRCs have so far only been described in germline *CDH1*-mutation-related DGC and have not been described in the noninvolved stomach of patients with sporadic SRCC. Confirmation of precursors of SRCC by an independent histopathologist is recommended since various benign "signet cell-like changes" may mimic these lesions [47]. In most specimens, there are often only a low number or no intraepithelial SRCCs at all identified in contrast to numerous T1a foci.

The surrounding gastric mucosa of these prophylactic gastric specimens is often without significant abnormalities. Background changes that are described include mild chronic gastritis, foveolar hyperplasia with tufting of the surface epithelium and globoid change with clear changes of the superficial epithelium [32, 59]. Intestinal metaplasia, atrophy, dysplasia, and infection with *Helicobacter pylori* are very rarely observed.

Gastrectomy with Curative Intent for Advanced HDGC

Advanced HDGCs often present as linitis plastica with increased gastric wall thickness, corresponding to diffuse infiltration of all layers of the stomach wall by cancer cells indistinguishable from linitis plastica in sporadic cases of DGC, but cases with

a localized tumor do occur as well. The predominant histological pattern is a poorly cohesive carcinoma with only a few or no classic SRC morphology, sometimes with a mucinous or (micro-) tubular component particularly when present in lymph node metastasis. These GCs cannot be discriminated based on histology basis alone from advanced sporadic GC. However, if there are in situ lesions, pagetoid spread of SRCs or multiple intramucosal SRCC lesions at distance from the tumor bulk, these are important clues in favor of CDH1-related GC. In situ SRCC and pagetoid spread lesions have not been described so far in sporadic SRC/diffuse type GC [59].

Immunohistochemical Profile of HDGC and Its Relationship with *CDH1* Mutations

Consistent with the bi-allelic *CDH1* inactivation and consequent E-cadherin loss of function, E-cadherin protein expression by immunohistochemistry (IHC) is almost always abnormal in HDGC, in contrast to the normal complete membranous expression in adjacent normal (nontumoral) epithelium [44, 50–52, 59, 63, 64, 67–69]. Aberrant E-cadherin staining patterns include absence of immunoreactivity as well as reduced membranous, "dotted", and cytoplasmic staining. The "dotted" staining pattern is probably due to the persistence of E-cadherin nonfunctional domains in the Golgi apparatus [50]. Abnormal immunoreactivity of E-cadherin has been described in precursor lesions (in situ SRCC and pagetoid spread of SRCs) as well as in early or advanced carcinomas, suggesting that the inactivation of E-cadherin is probably a key initiating event in HDGC tumorigenesis [59]. Moreover, normal immunoreactivity of the gastric mucosa between lesions suggests a clonal origin of the individual cancer foci.

One report [50] described abnormal patterns of expression of both α- and β-catenin in early HDGCs, suggesting that the absence of a normal E-cadherin protein may lead to the disruption of the cell-cell adhesion complex. Furthermore, β-actin, p120 catenin, and Lin7 were shown to be reduced or absent in HDGC in another study [68].

In 2010, da Cunha et al. [70] investigated the expression of v6-containing CD44 isoforms (CD44v6), in the process of malignant transformation of gastric mucosa comparing precursor lesions and advanced sporadic and hereditary GCs. In the three HDGC cases from *CDH1* germline mutation carriers, a simultaneous loss of E-cadherin expression and overexpression of CD44v6 was observed, and CD44v6 was proposed to be a putative biomarker of early invasive intramucosal HDGC [70].

Based on prophylactic gastrectomy specimens, the microscopic foci of intramucosal SRCs are scattered throughout the stomach of *CDH1* germline mutation carriers, and represent early and asymptomatic lesions that have the potential to progress to aggressive and widely invasive carcinomas. However, few studies describe the immunohistochemical profile of these lesions [67, 68].

In general, early HDGCs were described as low-proliferative lesions, with few mitotic cells and low numbers of cells with Ki-67 expression, while advanced HDGCs showed many more Ki-67 positive cells [67, 68]. Ki-67 expression was observed in the small and less differentiated SRCs located at the base of larger intramucosal lesions [68]; however, in another study, those small and less differentiated SRCs were described as having a low proliferative index, similar to the superficial and more differentiated cells [67].

Since the small and dedifferentiated tumor cells that constitute the bulk of advanced HDGC and the deep layer of early HDGC display a morphology "reminiscent of mesenchymal cells," Humar et al. [68] hypothesized that epithelial-mesenchymal transition (EMT) mediates the progression from early to advanced HDGC. The activated kinase c-Src, a well characterized EMT inducer [71] and its downstream targets, such as fibronectin, p-Fak, and p-Stat3 were not expressed in small intramucosal foci of *SRCCs*, while the immunoreactivity was observed in dedifferentiated neoplastic cells in larger intramucosal lesions, and in advanced HDGCs with increased depth of invasion. These findings, however, could not be confirmed by Barber et al. [67]. These authors did not observe differences in immunoreactivity between smaller and larger intramucosal SRCCs. Barber et al. [67] investigated immunoreactivity of cytokeratins (CK) 8/18 and vimentin by dual-label immunofluorescence, and their results failed to demonstrate the evidence of EMT. CK expression in both differentiated and dedifferentiated SRCCs had also been described in previous studies [63, 68]. Moreover, N-cadherin, an EMT marker with increased expression in the presence of EMT [72] was not observed in intramucosal *foci* of SRC [51]. In conclusion, the role of EMT in the development of aggressive and widely invasive HDGC from early and indolent microscopic foci is uncertain and further analysis of these lesions is necessary to understand the molecular events required for the progression from indolent intramucosal lesions to widely invasive carcinomas.

The cell differentiation pattern of HDGC has been also investigated: Humar et al. [68] described MUC5A expression in differentiated, large SRCs at the surface of gastric mucosa, and MUC6 expression in poorly differentiated cells, at the neck zone, whereas Oliveira et al. [50] observed a widespread expression of gastric differentiation markers (MUC1, MUC5AC, and TTF1) within the lesions.

Another molecular pathway that has been explored in HDGC is the relationship between the disruption of apical-basal cell polarity induced by loss of functional E-cadherin and the resistance of cancer cells to anoikis, a particular form of programmed cell death that is triggered by the loss of cell-cell and cell-matrix normal interactions. The group of Raquel Seruca [73, 74] developed an in vitro model to test the pathogenicity of *CDH1* germline missense mutations found in HDGC patients and observed that the loss of functional E-cadherin renders cells more resistant to Taxol-mediated apoptotic stimuli and that an interplay exists between loss of E-cadherin and gain of the anti-apoptotic protein Bcl-2 activity, probably through the aberrant activation of Notch-1 [73, 74]. Such in vitro findings were supported by the Bcl-2 cytoplasmic immunoreactivity found in one case of a primary tumor harboring one of the mutations analyzed in the in vitro model [74].

New Insights in Morphological, Immunohistochemical, and Genetic Profile of HDGC

As HDGC encompasses a spectrum of precursor and invasive lesions, as described above, and the molecular events that occur in early and advanced carcinomas have not yet been clearly elucidated, we have recently investigated the relationship between the morphology of these lesions and the immunoexpression of biomarkers of cell-adhesion, proliferation, anoikis, and EMT (unpublished data). Moreover, we have explored the immunoexpression of a putative biomarker of cancer cell stemness, ALDH1A, a cytosolic protein that catalyzes the oxidation of endogenous and exogenous aldehydes in the equivalent carboxylic acids and their functions are fundamental in physiological processes, including proliferation, survival, differentiation, and detoxification. ALDH1A has been described as a biomarker of both normal progenitor and stem cells (hematopoietic, mesenchymal, neural, mammary, prostate, and gastrointestinal lineages) and cancer stem cells (CSCs), including head and neck, breast, prostate, ovarian, lung, hepatic, pancreatic, bladder, and colon cancers [75]. Moreover, high ALDH1A expression has been associated with adverse prognosis in breast, lung, serous ovarian, pancreatic, bladder, prostate, and oesophageal cancer [75]. With regard to GC, the findings are still conflicting and inconclusive [76–82] and, to our knowledge, no previous studies have investigated ALDH1A expression in HDGC and its precursor lesions, to assess the validity of this putative CSC biomarker in this specific setting.

We have recently undertaken a study including twenty-one lesions (from 17 surgical specimens belonging to 12 *CDH1*-related HDGC families), that encompassed 12 intramucosal carcinomas (pT1a) and 9 widely invasive carcinomas (pT > 1). The cases were reviewed by an expert pathologist in HDGC (FC) and were analyzed by IHC for E-cadherin (clone 4A2C7), Ki-67 (clone 30-9), Bcl-2 (clone 124), p53 (clone 318-6-11), pSrc (clone Y416), and ALDH1A (clone EP1933Y). Furthermore, the study included one case of endoscopic submucosal dissection (ESD) and four biopsy specimens, three of them corresponding to preoperative specimens of patients submitted to surgery, and two obtained from distinct HDGC families, both harboring the same *CDH1* mutation. The cases were retrieved from the Department of Pathology, Radboud university medical centre, Nijmegen (The Netherlands), the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York (USA), the Department of Histopathology and Molecular Pathology, Leeds Teaching Hospitals NHS Trust, Leeds (UK), the Department of Histopathology, Cambridge University Hospitals NHS Trust, Cambridge (UK) and the Department of Pathology, Centro Hospitalar de São João, Porto (Portugal). The morphological phenotype of these lesions included a variable spectrum of precursor, early and advanced lesions, namely: (1) precursor lesions (pTis), including in situ SRCC and pagetoid spread of SRCs; (2) intramucosal HDGC (pT1a), showing typical SRC morphology (“indolent phenotype”); and (3) advanced HDGC (pT > 1) composed of a mixture of SRCs and poorly cohesive, pleomorphic, and bizarre cells (“aggressive phenotype”). Interestingly, all these lesions were observed in one case in different locations of the same surgical specimen, as shown in Fig. 18.1.

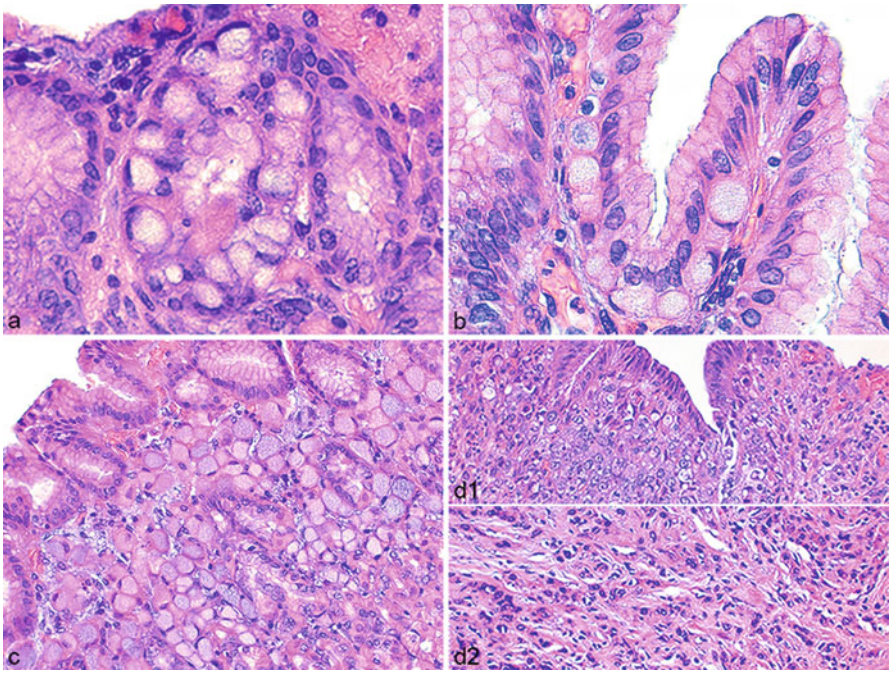

Fig. 18.1 Morphological spectrum of precursor, intramucosal, and widely invasive lesions of Hereditary Diffuse Gastric Carcinoma (HDGC) identified in one surgical specimen: (**a**) In situ (intraepithelial) signet ring cell (SRC) carcinoma (original magnification 400×). (**b**) Pagetoid spread of SRCs (original magnification 400×). (**c**) Intramucosal HDGC (pT1a) showing typical SRC morphology ("indolent" phenotype) (original magnification 200×). (**d1**) Intramucosal component of advanced HDGC (pT > 1), showing a mixture of signet ring and pleomorphic cells (original magnification 200×). (**d2**) Intramural component of advanced HDGC (pT > 1), showing pleomorphic cells (original magnification 200×). Hematoxylin and eosin (H&E)

All pTis and pT1a lesions showed "indolent" morphological features, and were negative for p53 and Ki-67 immunoexpression. In contrast, pT > 1 carcinomas were characterized by high Ki-67 proliferation index (89 %, $p < 0.01$) and p53 overexpression (56 %, $p < 0.01$) in the pleomorphic component of the tumors (Fig. 18.2). E-cadherin immunoexpression was abnormal in all precursor lesions, early and advanced SRCCs and showed a heterogeneous staining pattern, from absent or decreased membranous immunoreactivity to "dotted" pattern and cytoplasmic immunoreactivity (Fig. 18.3). Expression of ALDH1A and pSrc was higher in intramucosal carcinomas (100 and 58 %, respectively) compared to advanced carcinomas (44 %, $p < 0.01$ and 33 %, $p = 0.03$ respectively) (Fig. 18.4). Bcl-2 was expressed only in one case. The analysis of a putative relationship between biomarkers expression revealed a significant correlation between Ki-67 and p53 immunoreactivity ($p < 0.01$), while ALDH1A overexpression inversely correlated with Ki-67 and p53 overexpression ($p < 0.01$). We noted a tendency for pSrc overexpression to be associated with absence of Ki-67 and p53 immunoreactivity, but differences were not statistically significant.

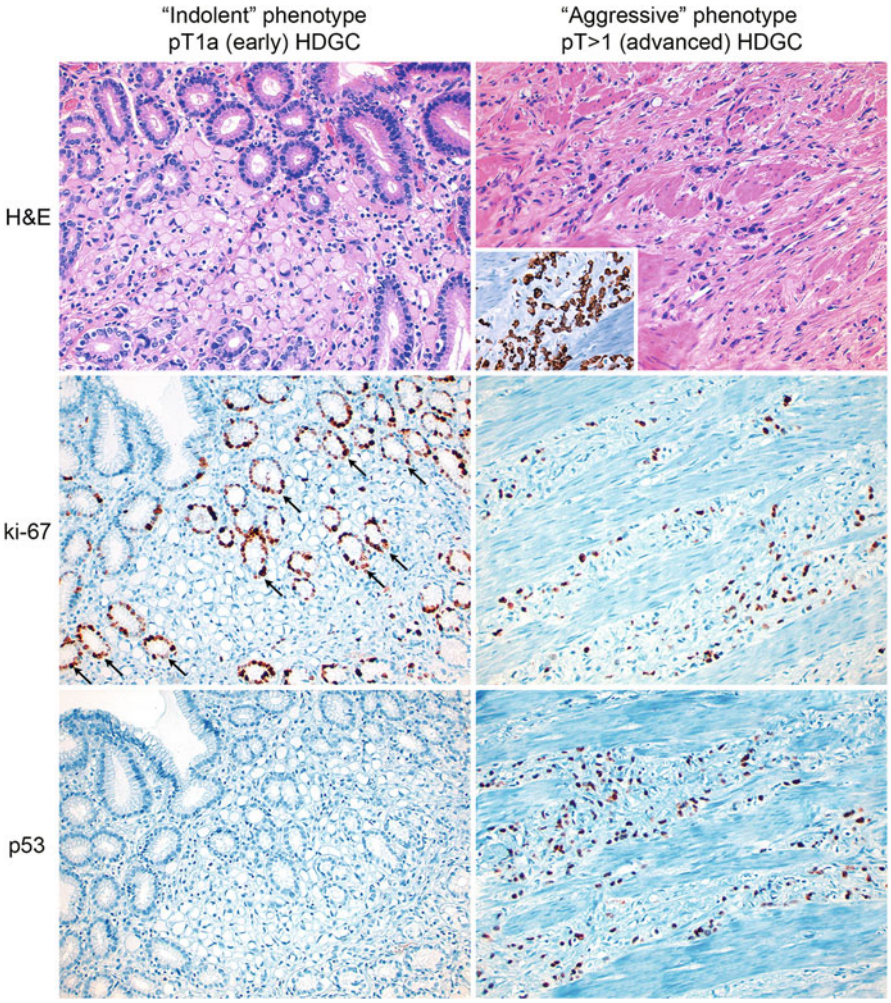

Fig. 18.2 Representative illustration of the "indolent" phenotype observed in intramucosal (pT1a) HDGC showing SRC morphology and absence of Ki-67 and p53 immunostaining (*left column*) and the "aggressive" phenotype in advanced (pT>1) HDGC characterized by pleomorphic cells and positivity for Ki-67 and p53 (*right column*). Note that Ki-67 is positive in the proliferative neck region of normal gastric glands (*arrows*), but negative in the intermingled SRCs of intramucosal HDGC. IHC, original magnification 200×. The *inset* in the right column shows immunohistochemical positivity of the neoplastic cells for cytokeratins (AE1/AE3)

The presence of ALDH1A immunoreactivity in normal gastric mucosa, and in all intramucosal SRCCs, together with the loss of such immunoreactivity in advanced HDGCs, exclude the possible role of ALDH1A as a biomarker of cancer cell stemness in HDGC. The studies of ALDH1A expression in epithelial cancers show that the percentage of ALDH1A positive tumor cells is strongly correlated with the level

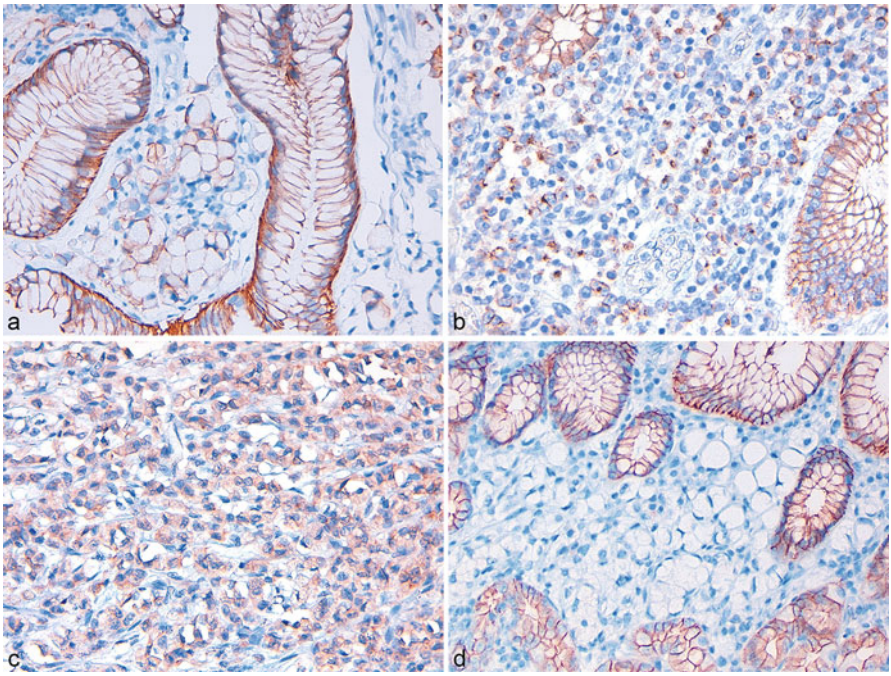

Fig. 18.3 E-cadherin immunostaining patterns that can be observed in precursor, intramucosal, and advanced HDGC lesions: (**a**) decreased membranous expression; (**b**) *dotted* staining; (**c**) faint cytoplasmic expression; (**d**) complete absence of immunostaining. IHC, original magnification 400×

of ALDH1A expression in the normal counterpart, suggesting that, as a CSC biomarker, ALDH1A may be useful only for tumors with a low level background expression of the protein in the normal counterpart [76]. GC may be added to the list of tumors in which ALDH1A is not useful as a CSC biomarker but, alternatively, may represent a marker of cellular differentiation.

A noteworthy study by Fricke et al. [83] investigated the relationship between E-cadherin and p53 gene mutation and p53, Ki67, and Bcl-2 immunoexpression in a series of 24 sporadic diffuse gastric carcinomas, 16 of which were positive for E-cadherin mutation. P53 overexpression was significantly more frequent in tumors without *CDH1* mutations than in GCs with *CDH1* mutations, while no correlation was found between Ki-67 immunoreactivity and the *CDH1* mutation status. Furthermore, *TP53* mutation was detected in 12.5 % of tumors without *CDH1* mutations and in 6.3 % in tumors with *CDH1* mutations, though the difference was not statistically significant. These findings may suggest that, in the sporadic GC setting, the presence of *CDH1* mutation can alter the accumulation of p53 protein.

To our knowledge, our study is the first to explore p53 immunoreactivity in the context of HDGC. In sporadic GC, p53 nuclear overexpression by IHC, was found both in intestinal and diffuse GCs and was correlated with tumor progression, poor

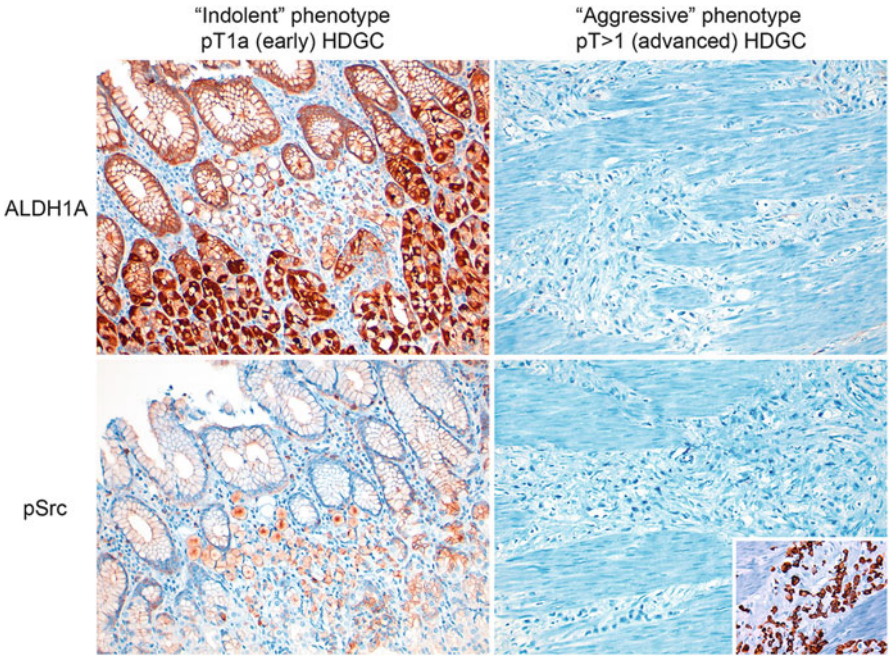

Fig. 18.4 Decreased expression of ALDH1A and pSrc from intramucosal to advanced carcinomas. IHC, original magnification 200×. The *inset* in the right column shows the immunohistochemical positivity of the neoplastic cells for cytokeratins (AE1/AE3)

prognosis and unfavorable response to therapy [83–88]. Moreover, NGS studies have identified *TP53* mutations as one of the most frequent alterations in GCs and *TP53* is as a candidate driver gene, especially in intestinal-type GC [53, 55–57, 89]. The demonstration of p53 nuclear accumulation in our study suggests that *TP53* may be a key gene involved in GC progression in the hereditary setting as well.

In the ESD and biopsy specimens from *CDH1* germline mutation carriers, both "indolent" and "aggressive" features of the neoplastic cells were observed (Fig. 18.5). Based on available evidence, the finding of GC with an "aggressive" phenotype in a screening biopsy performed in a *CDH1* germline mutation carrier should be taken as a predictive sign of widely invasive carcinoma and prompt staging and surgical intervention.

In the patients submitted to surgery, belonging to the 12 *CDH1*-related HDGC families, 11 different germline mutations in the *CDH1* gene were identified: 3 splice-site mutations; 3 frameshift, 1 missense, 1 missense/frameshift, and 3 nonsense mutations. Intramucosal carcinomas associated or not with widely invasive carcinomas were always found in carriers of different mutations, independently of the mutation site or its type. This observation likely reflects a disease mechanism and morphological phenotype that is characteristic of *CDH1* inactivation in the stomach, independent of the germline mutation, intimately associated with the first stages of HDGC development [59].

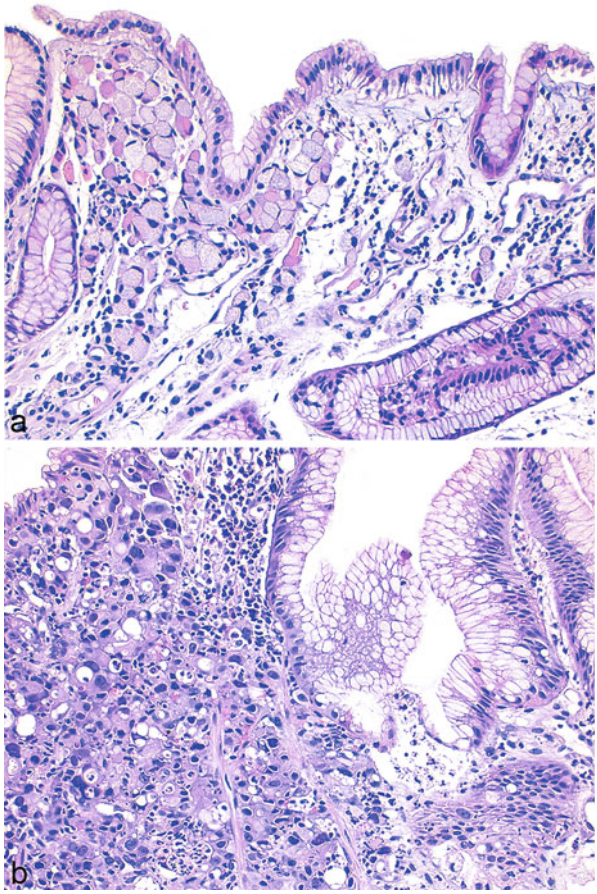

Fig. 18.5 Gastric carcinomas with "indolent" (**a**) and "aggressive" (**b**) features observed in endoscopic biopsies. Hematoxylin and eosin (H&E), original magnification 200×

We also analyzed two patients (19 and 20 years old) with metastatic and inoperable disease, from whom only biopsy specimens were available. These two cases, from 2 distinct HDGC families of different countries (Portugal and USA) were caused by the same missense/frameshift mutation c.1901C>T (p.Ala634Val), the GCs displayed an "aggressive" morphological phenotype and had significantly higher expression of Ki-67 and p53, compared to the cases from which surgical specimens were available ($p = 0.04$ and $p = 0.03$, respectively). Hence the c.1901C>T mutation deserves further analysis for its potential association with aggressive clinical behavior.

The appearance of early HDGC lesions that may evolve to widely invasive carcinomas is thought to be triggered in the stomach by somatic inactivation of the wild-type *CDH1* allele [49, 51, 52]. However, bi-allelic *CDH1* gene inactivation does not invariably lead to complete loss of E-cadherin protein expression. In fact, E-cadherin protein expression, as detected by IHC, can be maintained independent of *CDH1* germline mutation and HDGC tumor stage. Similar observations in HDGC

invasive carcinomas have been documented in several studies [51, 52]. Thus, IHC analysis of E-cadherin in HDGC lesions (early and advanced) is neither a valid marker to detect complete *CDH1* gene inactivation nor can it be used to predict whether an early lesion will evolve to invasive cancer.

Our data provide evidence that intramucosal (pT1a) and widely invasive HDGC carcinomas (pT>1) differ in their IHC expression of Ki-67 and p53, and that the expression of these markers in such lesions is independent of the type and site of the underlying *CDH1* germline mutation. In particular, it was observed that all early intramucosal lesions, regardless of germline mutation context, display characteristic SRC morphology and lack both Ki-67 and p53 expression, while most invasive carcinomas display a pleomorphic phenotype characterized by Ki-67 and p53 expression. These results show for the first time the proliferative nature of invasive HDGC and its association with abnormal p53 expression, as part of the progression molecular profile of HDGC tumors.

The finding of increased proliferation associated with p53 positivity in invasive and pleomorphic HDGC cells is of even greater importance when considering data from available mouse models of diffuse gastric cancer [90–93]. Out of three mouse models, the double conditional knockout in which both *CDH1* and *TP53* were specifically inactivated was the most efficient in producing diffuse gastric cancers by far [92]. These murine tumors were mainly composed of poorly differentiated cells and SRCs, similar to those in human advanced HDGC. Carcinoma developed within 12 months with 100 % penetrance. This mouse model mimics the human disease closely, and as demonstrated in the present study, very likely also recapitulates the genetic progression of *CDH1*-related HDGC in humans.

Conclusions and Practical Points

- Germline *CDH1* mutations are the most important cause for HDGC and give an increased risk of both DGC as well as LBC. Germline *CTNNA1* mutations were described in three families with diagnoses of DGC. There is limited data on other susceptibility genes for HDGC, including *MAP3K6,* and their role in GC remains to be determined.
- The updated *CDH1* testing criteria 2015 include families with (1) two or more patients with GC, one confirmed DGC; (2) DGC before the age of 40; (3) families with diagnoses of both DGC and LBC, one before the age of 50.
- Given the high mortality associated with invasive DGC, prophylactic total gastrectomy is advised for individuals with pathogenic *CDH1* mutations.
- Standardized endoscopic surveillance in experienced centers, preferably in a research setting, is recommended for those opting not to have gastrectomy at the current time, those with *CDH1* variants of uncertain significance and those that fulfill HDGC criteria but without germline *CDH1* mutations.
- Characteristic lesions in HDGC are tiny microscopic intramucosal foci of typical SRCs, in situ SRCC, and pagetoid spread of SRCs.

- Intramucosal SRCCs are invasive lesions that may remain indolent for an uncertain period of time; no metastatic disease has been described in prophylactic gastrectomies of *CDH1* mutation carriers with the diagnosis of exclusively intramucosal DGC.

New Hypotheses and Further Research Directions

- It remains unanswered how long early lesions of HDGC remain indolent, and how to predict their progression to widely invasive carcinomas.
- Intramucosal HDGCs present with an "indolent" phenotype (SRCs; Ki67–; p53–), while advanced carcinomas display an "aggressive" phenotype (pleomorphic cells; Ki67+; p53+). This is the first evidence of phenotypic heterogeneity in HDGC lesions, which may help define prognostic biomarkers of progression from indolent to widely invasive carcinomas.
- One of the first alterations in HDGC is inactivation of the wild-type *CDH1* allele. Other driver alterations in tumor suppressor genes and oncogenes that may play significant roles in the progression of DGC remain to be clarified.
- The causative germline mutations in patients with HDGC but without germline *CDH1* mutation remain unclear. It is likely that more HDGC-associated genes will soon be discovered using NGS methodologies. The management will be difficult until a larger number of mutation-positive families have been studied.
- Population-based germline interrogation of potential gene mutations and single nucleotide polymorphism in familial clusters may also generate noteworthy findings.

References

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F., GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. http://globocan.iarc.fr. Accessed on day/month/year, 2012.
2. Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO classification of tumours of the digestive system. IARC Sci Publ 2010; 4th ed., 2010.
3. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
4. Kawasaki K, et al. Family history of cancer in Japanese gastric cancer patients. Gastric Cancer. 2007;10(3):173–5.
5. La Vecchia C, et al. Family history and the risk of stomach and colorectal cancer. Cancer. 1992;70(1):50–5.
6. Hemminki K, Sundquist J, Ji J. Familial risk for gastric carcinoma: an updated study from Sweden. Br J Cancer. 2007;96(8):1272–7.

7. Carneiro F. Pathology of hereditary gastric cancer. In: Corso G, Roviello F, editors. Spotlight on familial and hereditary gastric cancer. Dordrecht: Springer; 2013. p. 141–56.
8. Caldas C, et al. Familial gastric cancer: overview and guidelines for management. J Med Genet. 1999;36(12):873–80.
9. Worthley DL, et al. Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS): a new autosomal dominant syndrome. Gut. 2012;61(5):774–9.
10. Oliveira C, et al. Familial gastric cancer: genetic susceptibility, pathology, and implications for management. Lancet Oncol. 2015;16(2):e60–70.
11. Park YJ, Shin KH, Park JG. Risk of gastric cancer in hereditary nonpolyposis colorectal cancer in Korea. Clin Cancer Res. 2000;6(8):2994–8.
12. Capelle LG, et al. Risk and epidemiological time trends of gastric cancer in Lynch syndrome carriers in the Netherlands. Gastroenterology. 2010;138(2):487–92.
13. Sereno M, et al. Gastric tumours in hereditary cancer syndromes: clinical features, molecular biology and strategies for prevention. Clin Transl Oncol. 2011;13(9):599–610.
14. Keller G, et al. Germline mutations of the E-cadherin(CDH1) and TP53 genes, rather than of RUNX3 and HPP1, contribute to genetic predisposition in German gastric cancer patients. J Med Genet. 2004;41(6):e89.
15. Oliveira C, et al. E-Cadherin (CDH1) and p53 rather than SMAD4 and Caspase-10 germline mutations contribute to genetic predisposition in Portuguese gastric cancer patients. Eur J Cancer. 2004;40(12):1897–903.
16. Masciari S, et al. Gastric cancer in individuals with Li-Fraumeni syndrome. Genet Med. 2011;13(7):651–7.
17. Iwama T, Mishima Y, Utsunomiya J. The impact of familial adenomatous polyposis on the tumorigenesis and mortality at the several organs. Its rational treatment. Ann Surg. 1993;217(2):101–8.
18. Lynch HT, et al. FAP, gastric cancer, and genetic counseling featuring children and young adults: a family study and review. Fam Cancer. 2010;9(4):581–8.
19. Giardiello FM, Trimbath JD. Peutz-Jeghers syndrome and management recommendations. Clin Gastroenterol Hepatol. 2006;4(4):408–15.
20. Giardiello FM, et al. Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology. 2000;119(6):1447–53.
21. van Lier MG, et al. High cancer risk and increased mortality in patients with Peutz-Jeghers syndrome. Gut. 2011;60(2):141–7.
22. Howe JR, Mitros FA, Summers RW. The risk of gastrointestinal carcinoma in familial juvenile polyposis. Ann Surg Oncol. 1998;5(8):751–6.
23. Pollock J, Welsh JS. Clinical cancer genetics: part I: gastrointestinal. Am J Clin Oncol. 2011;34(3):332–6.
24. Jakubowska A, et al. BRCA2 gene mutations in families with aggregations of breast and stomach cancers. Br J Cancer. 2002;87(8):888–91.
25. Friedenson B. BRCA1 and BRCA2 pathways and the risk of cancers other than breast or ovarian. MedGenMed. 2005;7(2):60.
26. Jones EG. Familial gastric cancer. N Z Med J. 1964;63:287–96.
27. Guilford P, et al. E-cadherin germline mutations in familial gastric cancer. Nature. 1998;392(6674):402–5.
28. Berx G, et al. Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). Genomics. 1995;26(2):281–9.
29. Berx G, et al. Mutations of the human E-cadherin (CDH1) gene. Hum Mutat. 1998;12(4):226–37.
30. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci. 2008;65(23):3756–88.
31. Suriano G, et al. Characterization of a recurrent germ line mutation of the E-cadherin gene: implications for genetic testing and clinical management. Clin Cancer Res. 2005;11(15):5401–9.
32. Oliveira C, Seruca R, Carneiro F. Genetics, pathology, and clinics of familial gastric cancer. Int J Surg Pathol. 2006;14(1):21–33.

33. Kaurah P, et al. Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. JAMA. 2007;297(21):2360–72.
34. Benusiglio PR, et al. CDH1 germline mutations and the hereditary diffuse gastric and lobular breast cancer syndrome: a multicentre study. J Med Genet. 2013;50(7):486–9.
35. Hansford S, Kaurah P, Li-Chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S, Lum A, Talhouk A, Baker-Lange K, Richardson S, Lewis I, Lindor NM, Pennell E, MacMillan A, Fernandez B, Keller G, Lynch H, Shah SP, Guilford P, Gallinger S, Corso G, Roviello F, Caldas C, Oliveira C, Pharoah PD, Huntsman DG. Hereditary diffuse gastric cancer syndrome CDH1 mutations and beyond. JAMA Oncol. 2015;1(1):23–32.
36. Rimm DL, et al. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. Proc Natl Acad Sci U S A. 1995;92(19):8813–7.
37. Koslov ER, et al. Alpha-catenin can form asymmetric homodimeric complexes and/or heterodimeric complexes with beta-catenin. J Biol Chem. 1997;272(43):27301–6.
38. Vasioukhin V, et al. Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. Cell. 2001;104(4):605–17.
39. Majewski IJ, et al. An alpha-E-catenin (CTNNA1) mutation in hereditary diffuse gastric cancer. J Pathol. 2013;229(4):621–9.
40. Jakubowska A, et al. A high frequency of BRCA2 gene mutations in Polish families with ovarian and stomach cancer. Eur J Hum Genet. 2003;11(12):955–8.
41. Moran A, et al. Risk of cancer other than breast or ovarian in individuals with BRCA1 and BRCA2 mutations. Fam Cancer. 2012;11(2):235–42.
42. Risch HA, et al. Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. Am J Hum Genet. 2001;68(3):700–10.
43. Johannsson O, et al. Incidence of malignant tumours in relatives of BRCA1 and BRCA2 germline mutation carriers. Eur J Cancer. 1999;35(8):1248–57.
44. Gaston D, et al. Germline mutations in MAP3K6 are associated with familial gastric cancer. PLoS Genet. 2014;10(10):e1004669.
45. Fitzgerald RC, et al. Hereditary diffuse gastric cancer: updated consensus guidelines for clinical management and directions for future research. J Med Genet. 2010;47(7):436–44.
46. Oliveira C, et al. Germline CDH1 deletions in hereditary diffuse gastric cancer families. Hum Mol Genet. 2009;18(9):1545–55.
47. Van der Post RS, Vogelaar IP, Carneiro F, Guilford P, Huntsman D, Hoogerbrugge N, Caldas C, Chelcun Schreiber KE, Hardwick R, Ausems MG, Bardram L, Benusiglio PR, Bisseling TM, Blair V, Bleiker E, Boussioutas A, Cats A, Coit D, DeGregorio L, Figueiredo J, Ford JM, Heijkoop E, Hermens R, Humar B, Kaurah P, Keller G, Lai J, Ligtenberg MJL, O'Donovan M, Oliveira C, Pinheiro H, Ragunath K, Rasenberg E, Richardson S, Roviello F, Schackert HK, Seruca R, Taylor A, Ter Huurne A, Tischkowitz M, Tjon S, Joe A, Van Dijck B, Van Grieken NC, van Hillegersberg R, Van Sandick JW, Vehof R, Van Krieken JH, Fitzgerald RC. Hereditary diffuse gastric cancer: updated clinical guidelines with an emphasis on germline CDH1 mutation carriers. J Med Genet. 2015;52(6):361–74.
48. Lim YC, et al. Prospective cohort study assessing outcomes of patients from families fulfilling criteria for hereditary diffuse gastric cancer undergoing endoscopic surveillance. Gastrointest Endosc. 2014;80(1):78–87.
49. Grady WM, et al. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet. 2000;26(1):16–7.
50. Oliveira C, et al. Intragenic deletion of CDH1 as the inactivating mechanism of the wild-type allele in an HDGC tumour. Oncogene. 2004;23(12):2236–40.
51. Barber M, et al. Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer. J Pathol. 2008;216(3):295–306.
52. Oliveira C, et al. Quantification of epigenetic and genetic 2nd hits in CDH1 during hereditary diffuse gastric cancer syndrome progression. Gastroenterology. 2009;136(7):2137–48.

53. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202–9.
54. Lee YS, Cho CY, Lee GK, Lee S, Kim YW, Jho S, Kim HM, Hong SH, Hwang JA, Kim SY, Hong D, Choi IJ, Kim BC, Kim BC, Kim CH, Choi H, Kim Y, Kim KW, Kong G, Kim HL, Bhak J, Lee SH, Lee JS. Genomic profile analysis of diffuse-type gastric cancers. Genome Biol. 2014;15(4):R55.
55. Wang K, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014;46(6):573–82.
56. Wang K, et al. Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nat Genet. 2011;43(12):1219–23.
57. Zang ZJ, et al. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet. 2012;44(5):570–4.
58. Liang H, Kim YH. Identifying molecular drivers of gastric cancer through next-generation sequencing. Cancer Lett. 2013;340(2):241–6.
59. Carneiro F, et al. Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening. J Pathol. 2004;203(2):681–7.
60. Rogers WM, et al. Risk-reducing total gastrectomy for germline mutations in E-cadherin (CDH1): pathologic findings with clinical implications. Am J Surg Pathol. 2008;32(6):799–809.
61. Charlton A, et al. Hereditary diffuse gastric cancer: predominance of multiple foci of signet ring cell carcinoma in distal stomach and transitional zone. Gut. 2004;53(6):814–20.
62. Shaw D, et al. Chromoendoscopic surveillance in hereditary diffuse gastric cancer: an alternative to prophylactic gastrectomy? Gut. 2005;54(4):461–8.
63. Huntsman DG, et al. Early gastric cancer in young, asymptomatic carriers of germ-line E-cadherin mutations. N Engl J Med. 2001;344(25):1904–9.
64. Chun YS, et al. Germline E-cadherin gene mutations: is prophylactic total gastrectomy indicated? Cancer. 2001;92(1):181–7.
65. Fujita H, et al. Endoscopic surveillance of patients with hereditary diffuse gastric cancer: biopsy recommendations after topographic distribution of cancer foci in a series of 10 CDH1-mutated gastrectomies. Am J Surg Pathol. 2012;36(11):1709–17.
66. Blair V, et al. Hereditary diffuse gastric cancer: diagnosis and management. Clin Gastroenterol Hepatol. 2006;4(3):262–75.
67. Barber ME, et al. Histopathological and molecular analysis of gastrectomy specimens from hereditary diffuse gastric cancer patients has implications for endoscopic surveillance of individuals at risk. J Pathol. 2008;216(3):286–94.
68. Humar B, et al. Destabilized adhesion in the gastric proliferative zone and c-Src kinase activation mark the development of early diffuse gastric cancer. Cancer Res. 2007;67(6):2480–9.
69. Norton JA, et al. CDH1 truncating mutations in the E-cadherin gene: an indication for total gastrectomy to treat hereditary diffuse gastric cancer. Ann Surg. 2007;245(6):873–9.
70. da Cunha CB, et al. De novo expression of CD44 variants in sporadic and hereditary gastric cancer. Lab Invest. 2010;90(11):1604–14.
71. Guarino M. Src signaling in cancer invasion. J Cell Physiol. 2010;223(1):14–26.
72. Lee JM, et al. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol. 2006;172(7):973–81.
73. Ferreira AC, et al. E-cadherin impairment increases cell survival through Notch-dependent upregulation of Bcl-2. Hum Mol Genet. 2012;21(2):334–43.
74. Ferreira P, et al. Loss of functional E-cadherin renders cells more resistant to the apoptotic agent taxol in vitro. Exp Cell Res. 2005;310(1):99–104.
75. Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev. 2011;7(2):292–306.
76. Deng S, et al. Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS One. 2010;5(4):e10277.
77. Zhi QM, et al. Salinomycin can effectively kill ALDH(high) stem-like cells on gastric cancer. Biomed Pharmacother. 2011;65(7):509–15.

78. Katsuno Y, et al. Coordinated expression of REG4 and aldehyde dehydrogenase 1 regulating tumourigenic capacity of diffuse-type gastric carcinoma-initiating cells is inhibited by TGF-beta. J Pathol. 2012;228(3):391–404.
79. Wakamatsu Y, et al. Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer. Pathol Int. 2012;62(2):112–9.
80. Wu C, et al. Lgr5 expression as stem cell marker in human gastric gland and its relatedness with other putative cancer stem cell markers. Gene. 2013;525(1):18–25.
81. Takahashi H, et al. A combination of nuclear beta-catenin and atypical scores as useful diagnostic markers for borderline malignancy of gastric tumours. Histopathology. 2014;65(6):828–38.
82. Xiao-shan Li QX, Xiang-yang F, Wei-sheng L. ALDH1A1 overexpression is associated with the progression and prognosis in gastric cancer. BMC Cancer. 2014;14:705.
83. Fricke E, et al. Relationship between E-cadherin gene mutation and p53 gene mutation, p53 accumulation, Bcl-2 expression and Ki-67 staining in diffuse-type gastric carcinoma. Int J Cancer. 2003;104(1):60–5.
84. Yildirim M, et al. Prognostic significance of p53 in gastric cancer: a meta-analysis. Asian Pac J Cancer Prev. 2015;16(1):327–32.
85. Busuttil RA, et al. Role of p53 in the progression of gastric cancer. Oncotarget. 2014;5(23):12016–26.
86. Teh M, Lee YS. An immunohistochemical study of p53 protein in the different histological subtypes of gastric carcinoma. Pathology. 1994;26(4):432–4.
87. Brito MJ, et al. Expression of p53 in early (T1) gastric carcinoma and precancerous adjacent mucosa. Gut. 1994;35(12):1697–700.
88. Ranzani GN, et al. p53 gene mutations and protein nuclear accumulation are early events in intestinal type gastric cancer but late events in diffuse type. Cancer Epidemiol Biomarkers Prev. 1995;4(3):223–31.
89. Kakiuchi M, et al. Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet. 2014;46(6):583–7.
90. Riethmacher D, Brinkmann V, Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proc Natl Acad Sci U S A. 1995;92(3):855–9.
91. Humar B, Blair V, Charlton A, More H, Martin I, Guilford P. E-cadherin deficiency initiates gastric signet-ring cell carcinoma in mice and man. Cancer Res. 2009;69(5):2050–6.
92. Shimada S, et al. Synergistic tumour suppressor activity of E-cadherin and p53 in a conditional mouse model for metastatic diffuse-type gastric cancer. Gut. 2012;61(3):344–53.
93. Mimata A, Fukamachi H, Eishi Y, Yuasa Y. Loss of E-cadherin in mouse gastric epithelial cells induces signet ring-like cells, a possible precursor lesion of diffuse gastric cancer. Cancer Sci. 2011;102(5):942–50.

Chapter 19
Helicobacter pylori, Cancer, and the Gastric Microbiota

Lydia E. Wroblewski and Richard M. Peek Jr.

Gastric Cancer

Gastric adenocarcinoma is the third leading cause of cancer-related death in the world, resulting in approximately 723,000 deaths in 2012, and the 5-year survival rate in the United States is less than 15 % [1–3].

The most common type of cancer that affects the stomach is adenocarcinoma, but lymphoma and leiomyosarcoma may also occur. Two distinct variants of gastric adenocarcinoma can be differentiated histologically; diffuse-type gastric cancer, which consists of individually infiltrating neoplastic cells that do not form glandular structures, and intestinal-type adenocarcinoma, which progresses through a series of well-defined histological steps [4]. Recent comprehensive molecular analysis of almost 300 primary gastric adenocarcinomas suggested a molecular classification dividing gastric cancer into four subtypes [5]. Cristescu et al. used gene expression data to classify gastric cancer into four molecular subtypes. The first are microsatellite unstable tumors (MSI), which occur in the antrum and possess the best overall prognosis with the lowest rate of reoccurrence. Tumor protein 53 (TP53)-active and TP53-inactive types have an intermediate prognosis, with the latter yielding worse prognosis than the former. Mesenchymal-like type forms the fourth subtype, and predicts the worst prognosis and highest frequency of recurrence [6].

The incidence of gastric adenocarcinoma in developed countries has significantly decreased over the past century, primarily due to a decline in intestinal-type adenocarcinomas in the distal stomach [7, 8]. Conversely, the incidence rates of proximal gastric adenocarcinomas as well as those originating within the gastroesophageal junction have been increasing in both the United States and Europe [9, 10].

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The strongest known risk factor for developing gastric adenocarcinoma is chronic infection with *H. pylori*. The degree to which *H. pylori* increases the risk for gastric adenocarcinoma can vary between studies and is likely dependent on several factors including patient age, selection of controls, and the site and stage of gastric cancer. In one study *H. pylori* infection accounted for 6.2 % of all cancers [11], and in another study the combined incidence of intestinal and diffuse-type gastric cancer in *H. pylori*-colonized individuals was reported to be approximately 3 %, compared with 0 % in uninfected persons [12]. To date it is not possible to accurately predict which infected individuals will develop gastric cancer.

H. pylori

H. pylori is a Gram-negative bacterial species that selectively colonizes the gastric epithelium. In 1994, *H. pylori* was recognized as a Type I carcinogen by the WHO, and chronic infection with this organism is the strongest known risk factor for distal gastric adenocarcinoma [13, 14]. *H. pylori* is usually acquired in childhood and in the absence of combined antibiotic therapy can persist for the lifetime of the host, despite the harsh gastric environment [15]. Interestingly, genetic studies indicate that *H. pylori* has colonized humans for at least 58,000 years [16], and approximately half of the world's population is infected with *H. pylori* leading some to speculate that *H. pylori* is an endogenous member of the gastric microbiota. Between 1 and 3 % of persons colonized with *H. pylori* develop gastric adenocarcinoma [17] and factors that play a role in the pathologic outcome of *H. pylori* infection are multifactorial, including strain-specific bacterial constituents, host genetic factors, alterations of the stem niche and host microbiota, and environmental influences including diet [18].

H. pylori Virulence Factors That Influence Gastric Pathogenesis

Bacterial virulence factors play a key role in determining the risk of developing gastric adenocarcinoma following colonization with *H. pylori*. One *H. pylori* virulence factor that clearly influences cancer risk is the *cag* pathogenicity island (*cag*-PAI), a 40-kB DNA insertion element containing genes which encode proteins that form a type IV bacterial secretion system (T4SS). The *cag* T4SS exports CagA from adherent *H. pylori* across the bacterial and epithelial membranes into host cells [19–22].

H. pylori strains that contain CagA are associated with a 5.8-fold increased risk of developing intestinal and diffuse gastric adenocarcinoma compared with uninfected persons. *H. pylori* strains that lack CagA induce only a 2.2-fold increased risk of developing distal gastric adenocarcinoma compared to uninfected persons

[23]. A meta-analysis of studies examining cancer risk suggests that *H. pylori* strains harboring CagA increase the risk of developing distal gastric adenocarcinoma twofold over the risk incurred by CagA-negative strains of *H. pylori* [24].

Following translocation, CagA can be tyrosine phosphorylated at N-terminal glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs. Four different EPIYA motifs (EPIYA-A, -B, -C, or –D) have been identified within CagA and can be used as indicators of pathologic outcome [25–27]. An elevated risk of developing gastric cancer is associated with an increased burden of CagA EPIYA-C sites [28], and strains that contain the EPIYA-D motif are associated with increased pathogenesis compared with strains harboring C-type CagA [25, 29]. Nonphosphorylated CagA also exerts effects within host cells that contribute to pathogenesis and has multiple effects on the apical-junctional complex. Specifically, unmodified CagA targets β-catenin, E-cadherin, the hepatocyte growth factor receptor c-Met, the phospholipase PLC-γ, the adaptor protein Grb2, and the kinase PAR1b/MARK2, leading to pro-inflammatory and mitogenic responses, disruption of cell-cell junctions, and loss of cellular polarity [30–37]. In addition, nonphosphorylated CagA also associates with the epithelial tight-junction scaffolding protein ZO-1, and the transmembrane protein junctional adhesion molecule (JAM)-A, leading to ineffective assembly of tight junctions in regions where *H. pylori* is attached [34]. In a CagA-independent manner *H. pylori* can also dysregulate the tight junction proteins occludin and claudin-7 and may alter barrier function [38, 39].

Another *H. pylori* constituent linked to the development of gastric cancer is VacA [40, 41]. VacA is a secreted toxin that causes multiple alterations in host gastric epithelial cells, including vacuolation, altered plasma and mitochondrial membrane permeability, autophagy, and apoptosis [40]. All strains of *H. pylori* contain *vacA*, but there are considerable differences in *vacA* sequences among strains. The regions of greatest diversity are localized to the 5′ region of the gene, which encodes the signal sequence and amino-terminus of the secreted toxin (allele types s1a, s1b, s1c, or s2), an intermediate region (allele types i1 or i2), and a mid-region (allele types m1 or m2) [42, 43]. Strains containing type s1, i1, or m1 alleles are strongly associated with gastric cancer [42, 44, 45]. New studies suggest the association between type i1 alleles and gastric cancer may even be stronger than the risk incurred by *vacA* s- or m-types, or even *cag* status [43, 46, 47].

Intriguing new insights suggest that VacA and CagA may counter-regulate each other to manipulate host cell responses. Specifically, CagA antagonizes VacA-induced apoptosis and activates a cell survival pathway mediated by MAPK and the antiapoptotic protein MCL1 [48]. It has recently been reported that the opposing effects of CagA and VacA may be cell lineage specific. In vivo lineage tracing of the gastric epithelium has demonstrated that Lgr5 (leucine-rich repeat-containing G protein-coupled receptor 5) positive cells are self-renewing, multipotent stem cells responsible for long-term renewal of the gastric epithelium [49]. In *H. pylori*-infected persons with gastric cancer the population of $Lgr5^+$ epithelial cells is expanded compared to uninfected persons with cancer. Furthermore, these $Lgr5^+$ epithelial cells are more susceptible to oxidative DNA damage than Lgr5-negative cells [50], indicating that *H. pylori* specifically targets $Lgr5^+$ epithelial cells.

In differentiated gastric epithelial cells, autophagy is induced in order to degrade intracellular CagA, and binding of VacA to the epithelial cell receptor LRP1 leads to a decrease in intracellular glutathione and allows accumulation of reactive oxygen species, which subsequently induced autophagy [51]. Interestingly, CagA was found to accumulate in gastric epithelial cells that express a stem cell marker, CD44 variant 9. These cancer stem-like cells are resistant to reactive oxygen species and as a result CagA is not degraded by autophagy. Collectively these data suggest that the bacterial oncoprotein CagA is able to persist in a subpopulation of host cells with progenitor-like features, which may confer long-term detrimental effects on the host that may lower the threshold for carcinogenesis [51].

Host and Environmental Factors That Influence Gastric Pathogenesis

Host polymorphisms also influence the propensity towards gastric cancer development. IL-1ß is a pro-inflammatory molecule that inhibits acid secretion and is increased within the gastric mucosa of *H. pylori*-infected persons. In the context of *H. pylori* infection, individuals with high-expressing IL-1ß polymorphisms have a significantly increased risk for hypochlorhydria, gastric atrophy, and distal gastric adenocarcinoma compared to individuals with genotypes that limit IL-1ß expression [52]. The combination of a more virulent strain of *H. pylori* in a genetically susceptible person further increases the risk of developing gastric cancer. Individuals harboring high-expressing IL-1ß polymorphisms who are infected with *H. pylori* *cagA*$^+$ or *vacA* s1-type strains have a 25-fold or 87-fold increase in risk, respectively, for developing gastric cancer compared to uninfected individuals [53]. Similar to IL-1ß, TNF-α is also a pro-inflammatory cytokine that inhibits acid secretion, and polymorphisms that increase TNF-α expression are also associated with augmenting the risk of developing gastric cancer and its precursors in the presence of *H. pylori* [54].

Environmental factors such as diet also increase the risk of developing gastric carcinoma. Diets high in salted, pickled, or smoked, or poorly preserved foods, those with a high meat content, and those with low fruit and vegetable content are most commonly associated with an increased risk for developing gastric cancer [55–61]. Within the context of *H. pylori* infection, high dietary salt intake and low iron levels are most highly associated with increased risk for developing gastric cancer [62–64].

To date, infection with *H. pylori* is the strongest identified risk factor for developing gastric cancer; however, human trials have indicated that other components of the gastric microbiota may influence gastric disease progression. In a 15-year follow-up study of 3365 subjects, it was reported that antibiotic therapy directed against *H. pylori* significantly reduced the incidence of gastric cancer. What is especially interesting about this study is that less than half of the individuals who

received antibiotics remained free of *H. pylori* at the 15-year follow-up [65]. This suggests that treatment with antibiotics may modify the non-*H. pylori* microbiota in such a way that the development of gastric cancer is attenuated.

The Human Gastric Microbiota in Gastric Pathogenesis

The acidic environment inherent to the stomach in combination with low levels of cultured bacteria from this site led to assumptions that the stomach was a somewhat sterile environment; however, data now show that the stomach harbors a large and diverse bacterial community with colonization densities ranging from 10^1 to 10^3 colony forming units/g [66]. Moreover, recent advancements in molecular techniques and computational analysis have provided evidence that the complex microbiota colonizing the gastric epithelium may influence gastric homeostasis and disease in combination with *H. pylori* [67].

H. pylori-negative individuals possess highly diverse gastric microbiomes (Fig. 19.1). Sequencing of 1833 bacterial clones from 23 gastric biopsy samples identified 128 phylotypes within 8 bacterial phyla; the 5 most abundant phyla were Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria [68, 69]. Interestingly, Bik et al. did not detect any *H. pylori*-induced alterations in the composition of the gastric microbiota, although *H. pylori* DNA was detected in 7 individuals who were considered to be *H. pylori* negative by traditional diagnostic technologies [68]. An independent study using tagged 454 pyrosequencing analysis of 3 *H. pylori*-negative gastric biopsy samples identified 262 phylotypes representing 13 phyla [70], supporting the notion of a highly diverse gastric microbiota despite substantial variability in the composition of the microbiota between individuals [68, 70]. In contrast, among *H. pylori*-infected individuals, *H. pylori* was found to be the single most abundant phylotype present in the stomach of persons testing positive for this organism [68, 70]. Among the three *H. pylori*-colonized persons tested, *H. pylori* accounted for 93–97 % of all sequence reads and only 33 phylotypes were detected; 229 fewer phylotypes than were detected in *H. pylori*-negative persons [70]. These data suggest that colonization with *H. pylori* greatly reduces the overall diversity of the gastric microbiota. In a more recent study using DNA microarrays to characterize the gastric microbiota in 12 corpus biopsy samples (8 of which were *H. pylori* positive), Maldonado-Contreras et al. detected 44 phyla with four dominant phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. *H. pylori* infection increased the relative abundance of non-*H. pylori*-Proteobacteria, Spirochaetes, and Acidobacteria while decreasing the relative abundance of Actinobacteria, Bacteroidetes, and Firmicutes, compared to patterns seen in uninfected stomachs [71]. In this study, *H. pylori* infection was found to account for 28 % of the variance in the microbiota; however, the bacterial communities in both *H. pylori*-negative and -positive individuals remained highly complex [71].

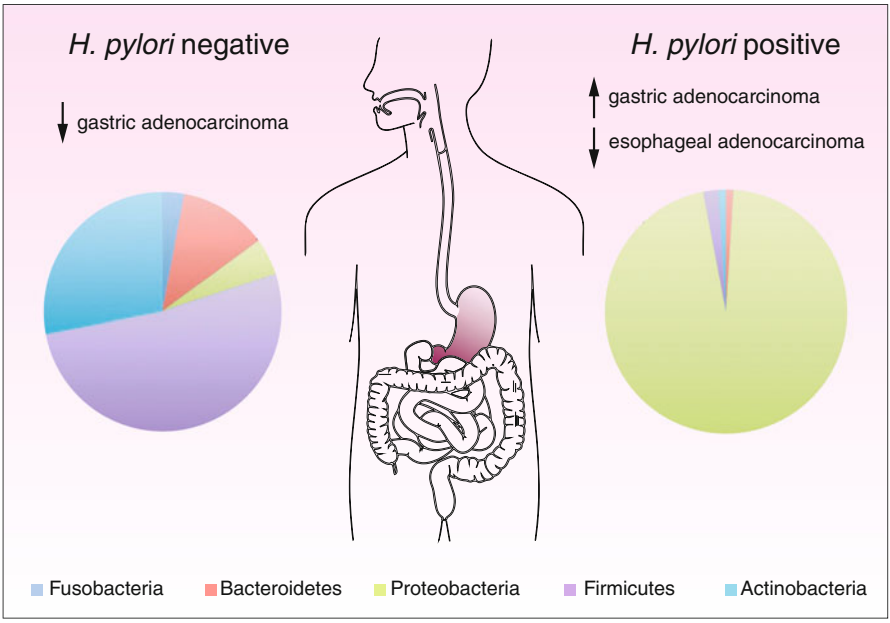

Fig. 19.1 Schematic representation showing the differences in the composition of the human gastric microbiota based on *H. pylori* status. *H. pylori*-negative individuals possess a highly diverse gastric microbiota and exhibit decreased risk of developing gastric adenocarcinoma when compared to *H. pylori* positive individuals who harbor a less diverse microbiota, possess an increased risk for developing gastric adenocarcinoma and concomitant decreased risk for developing esophageal adenocarcinoma

Currently, there are very few studies that have examined differences in microbial composition and outcomes of gastric cancer. One of the key steps in the histologic progression to intestinal-type gastric cancer is the development of atrophic gastritis, a condition that predisposes the stomach to an increase in gastric pH due to loss of parietal cells and overgrowth of non-*Helicobacter* microbiota [4]. A hypochlorhydric environment in the stomach facilitates colonization of other bacteria and may promote the progression towards gastric cancer. In a study focused on the microbiota in ten gastric cancer patients and 5 dysplastic controls using terminal restriction fragment length polymorphism (T-RFLP) in combination with 16S rRNA gene cloning and sequencing, Dicksved et al. found no significant differences between the composition of the gastric microbiota of patients with and without gastric cancer [72]. Specifically, the microbiota of patients with gastric cancer was as complex as the microbiota of dysplastic patients, with five bacterial phyla identified; Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. *H. pylori* was present in relatively low abundance and the microbiota was, instead, dominated by species of *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Prevotella* [72]. In a more recent study using pyrosequencing to compare the microbiota in gastric mucosa from persons with chronic gastritis, intestinal metaplasia, and gastric cancer, ten bacterial

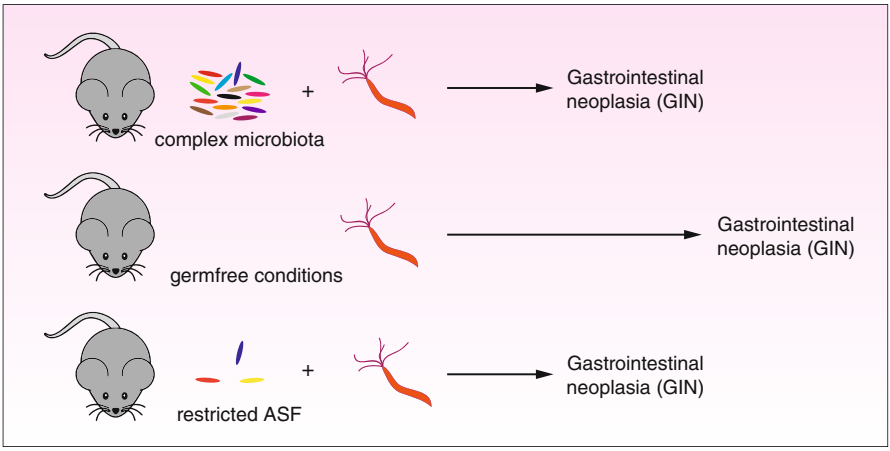

Fig. 19.2 Schematic representation showing the role of the gastric microbiota in the progression towards gastric neoplasia in the context of *H. pylori* infection. Gastrointestinal intraepithelial neoplasia (GIN) spontaneously developed in specific pathogen free (SPF) mice harboring a complex microbiota. In contrast, in germ-free mice, development of GIN in response to *H. pylori* (Hp) infection was over a year slower. In mice harboring a restricted microbiota containing only three species of commensal bacteria (restricted ASF), GIN developed at a rate indistinguishable from SPF mice [89, 92]. Interaction between *H. pylori* and the microbiota influences gastric disease progression

phyla were identified, suggesting the gastric microbiota is even more complex than previously thought [73]. Moreover, significant differences were observed in both the composition and diversity of the gastric microbiota along the distinct histological steps towards gastric cancer. Specifically, Bacilli and members of the Streptococcaceae family were significantly increased in gastric cancer samples compared with chronic gastritis and intestinal metaplasia samples and Epsilonproteobacteria and members of the Helicobacteraceae family were decreased [73].

An interesting dichotomy in disease outcome is that gastric colonization with *H. pylori* appears to confer protection against esophageal adenocarcinoma (Fig. 19.1) [74]. This may be due to *H. pylori*-induced hypochlorhydria as a result of loss of parietal cell function, especially in individuals who possess high expression IL-1ß polymorphisms (see Host factors that influence gastric pathogenesis for further details), or from loss of parietal cells in atrophic gastritis [67]. An alternative hypothesis is that perturbations in the gastric microbiota resulting from the absence of *H. pylori* may increase the propensity for an individual to develop esophageal adenocarcinoma [67].

In order to determine whether changes in the gastric microbiota play a role in the development of gastric cancer or are secondary to the changing gastric environment, further detailed molecular studies to define the composition of the gastric microbiota in well-characterized human populations, with and without gastric cancer, will need to be conducted. Studies of rodent model systems should help identify important drivers and modifiers of diseases related to the microbiome.

The Mongolian Gerbil Gastric Microbiota and Gastric Pathogenesis

The Mongolian gerbil has frequently been used to study *H. pylori*-induced disease and *H. pylori* infection in this model can lead to gastric adenocarcinoma without the co-administration of carcinogens [35, 75–77]. Similar to humans, gastric cancer develops in the distal stomach of gerbils, and another advantage of this model is that several *H. pylori* wild-type and mutant strains colonize well [78–80], thus allowing for the investigation of the role of virulence determinants on parameters of gastric injury. A drawback to this model is that Mongolian gerbils are outbred, which increases the variability of responses to any stimulus and does not allow for genetic manipulation (Fig. 19.3).

Currently, very little is known about the composition of the gerbil gastric microbiota, but most commonly represented phyla include Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes [81, 82]. Similar to mice, the genus *Lactobacillus* dominates the gastric microbiota of uninfected gerbils [81–83].

Sun et al. used molecular techniques to compare alterations in the gerbil gastric microbiota before and after 12 weeks of *H. pylori* infection [83]. Using temporal temperature gradient gel electrophoresis and pyrosequencing of gastric mucosal samples, Sun et al. reported that *Lactobacillus* was the dominant bacteria in the stomach of *H. pylori*-infected as well as in uninfected gerbils [83]. *Bacillus subtilis, Acinetobacter species, Pseudomonas species, Corynebacterium species, Enterococcus species, Paenibacillus species, Staphylococcus species,* along with unidentified bacteria, were also represented in the gerbil gastric microbiota [83]. In a longer-term study of uninfected and *H. pylori*-infected animals, quantitative PCR was used to track the relative abundance of 15 species of microbes in the gerbil stomach following

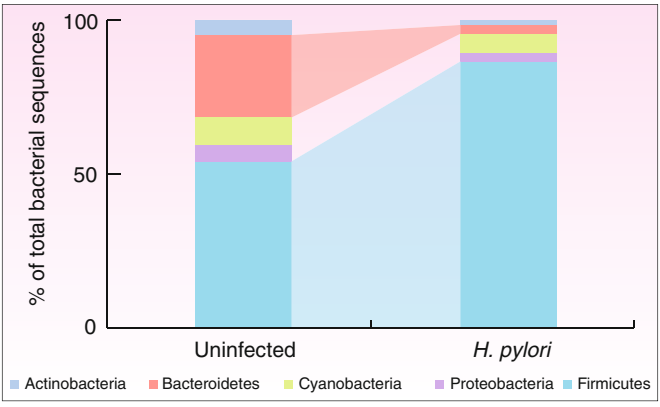

Fig. 19.3 Differences in the composition of the gastric microbiota in *H. pylori*-infected and uninfected mice. There are variations in the relative abundance of phyla in the stomach of *H. pylori*-infected and uninfected INS-GAS mice. *H. pylori* infection significantly increases the relative abundance of *Firmicutes* and decreases *Bacteroidetes* [89]

1 year of infection [84]. In uninfected gerbils, the most abundant genera were *Lactobacillus* and *Enterococcus,* followed by equivalent levels of *Atopobium* and *Clostridium*. In gerbils that were challenged and successfully colonized with *H. pylori*, the relative abundance of *Clostridium coccoides* increased when compared to uninfected gerbils. In gerbils that were challenged with *H. pylori* but not successfully colonized, the proportion of *C. coccoides*, *C. leptum*, and *Bifidobacterium* species was reduced when compared to noninfected gerbils [84]. Another recent analysis of the microbiota in gerbils with and without *H. pylori* infection revealed the presence of *Lactobacillus reuteri, Lactobacillus johnsonii,* and *Lactobacillus murinus* using genomic sequencing and interestingly these strains exerted an inhibitory effect on the growth of *H. pylori* in vitro [85]. The overall importance of differences in microbial composition and the development of gastric cancer; however, have not yet been determined in this model.

The Mouse Gastric Microbiota and Disease

Inbred mice with defined genotypes are another commonly used model of gastric carcinogenesis. In contrast to Mongolian gerbils, transgenic mice can be generated which allows for in-depth analyses of host responses. However, similar to the Mongolian gerbil model, standard inbred mice are frequently limited by their uncontrolled microbial diversity. Gnotobiotic animals are a powerful tool to be able to control the microbiome and add back individual or collections of microorganisms. To date, generation of germ-free gerbils has not been possible; however, gnotobiotic mice can be generated with any required gene mutation to test how genetic alterations in the host may be involved in establishing or controlling the microbiota. The limitations of this model are that it can be very expensive and specialized facilities and expertise are required, limiting their widespread use.

Using 16S rRNA gene cloning and a microarray-based Phylochip microbial profiling system, Rolig et al. identified 10,207 species groups in the mouse stomach and over 2000 of these were identified in all five mice that were analyzed. The Firmicutes phylum accounted for over 50 % of the isolates, with *Clostridia* being the most common class, followed by *Mollicutes* and *Bacilli* respectively. Members of the Bacteroidetes and Verrucomicrobia phyla were the second and third most common phyla, followed by Proteobacteria and Actinobacteria. Similar to what has been reported in the human stomach, the phylotypes with the most members were the *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* [86].

H. pylori induces chronic atrophic gastritis in the mouse stomach; however, bacteria other than *Helicobacter* species have also been found to induce gastritis in mice. Oral challenge of mice with *Acinetobacter lwoffii* in the absence of *H. pylori* can induce gastric inflammation and metaplastic changes similar to that induced by *H. pylori* [87]. The dominant genus in the uninfected mouse stomach is *Lactobacillus* [81, 82]; however, it is now becoming evident that despite mice having identical genetic backgrounds, their commercial source vendor can affect the composition of

the gastric microbial populations [86, 88]. Despite equal levels of colonization, C57BL/6 mice from two independent vendors developed different grades of inflammation in response to infection with *H. pylori*. Rolig et al. determined that different ratios of *Lactobacillus* species ASF360 and ASF361 were present in the gastric microbiota of mice obtained from two different vendors, and these variations accounted for the differences in inflammation and injury responses when challenged with *H. pylori* [86].

Infection of mice with *H. pylori* can alter the gastric microbiota, which appears to depend on the strain of mouse and duration of infection. In one study using Phylochip analysis, the microbiota of mice infected with *H. pylori* for 4 weeks was not significantly altered overall; however, the abundance of *Firmicutes, Bacteroidetes*, and *Proteobacteria* was decreased and of *Firmicutes* (class *Clostridia*), *Proteobacteria* (genus *Helicobacter*), and *Verrucomicrobia* was increased [86]. Perhaps not surprisingly, administration of antibiotics to mice prior to *H. pylori* infection dramatically altered the composition of the gastric microbiota, changing over 4400 species groups. Members of the *Firmicutes* phylum changed most profoundly and the severity of gastric inflammation in response to *H. pylori* infection was reduced. The inflammatory response was reversed when the gastric microbiota from antibiotic-naïve mice was transferred to mice given antibiotics and was comparable to the inflammatory response observed in an untreated normal mouse [86].

In specific pathogen free (SPF) female Balb/c mice, a 2-month infection with *H. pylori* was found to alter the gastric microbiota by reducing the number of *Lactobacillus* species and increasing bacterial diversity [89]. Of interest in this study is that immunizing mice with *Salmonella enterica* expressing *H. pylori* urease prevented *H. pylori*-induced changes in the gastric microbiota. It should be noted however that *H. pylori* colonization levels were two orders of magnitude lower in vaccinated mice compared to unvaccinated mice [89]. In contrast, studies in SPF C57BL/6 mice have produced conflicting results. In one study using T-RFLP analysis and culture, both acute and chronic infection of C57BL/6 mice with *H. pylori* did not cause significant shifts in the bacterial composition of the gastric microbiota [90].

In other studies using transgenic hypergastrinemic INS-GAS mice that are genetically predisposed to gastric cancer, chronic interaction between *H. pylori* and the gastric microbiota influenced disease progression [91]. In SPF INS-GAS mice harboring a complex microbiota, gastric cancer spontaneously developed [92, 93]. However, in germ-free INS-GAS mice, it took over a year longer for the development of gastric cancer [91] (Fig. 19.2). In addition, germ-free INS-GAS mice that were infected with *H. pylori* developed less severe lesions and were slower to progress to gastrointestinal intraepithelial neoplasia than *H. pylori*-infected SPF INS-GAS mice with a complex microbiota [91]. When the composition of the gastric microbiota was characterized using 454 sequencing of partial 16S ribosomal DNA amplicons, specific differences in phyla were observed between *H. pylori*-infected and uninfected SPF INS-GAS mice. A 12-week infection with *H. pylori* led to an expansion in the proportion of Firmicutes and decreased numbers of Bacteroidetes while causing an overall increase in species diversity [91]. A more recent study demonstrated that a

restricted microbiota containing only three species of commensal bacteria (ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus*, and ASF519 *Bacteroides species*) was sufficient to promote gastric neoplasia in *H. pylori*-infected INS-GAS mice to the same extent as observed in *H. pylori*-infected SPF INS-GAS mice [94].

Extragastric constituents of the microbiota may also influence outcomes of *H. pylori*-induced disease in mice. Co-infection of C57BL/6 mice with the enterohepatic *Helicobacter* species *H. bilis* or *H. muridarum* significantly attenuated *H. pylori*-induced gastric pathology despite chronic inflammation and efficient colonization of *H. pylori* [95, 96]. Mechanistically this was thought to be mediated through an attenuated T helper 1-associated IgG2c response [96]. In contrast, pre-existing infection with *H. hepaticus* increased *H. pylori*-induced gastric injury at 6 months of infection [95]. The mechanism was not thought to involve a T helper 1-type cell response but, was instead, thought to be mediated by a T helper 17-type cell response to the combined infection [95].

Interestingly, a study suggests that Helminth infections may prevent *H. pylori*-induced changes in the microbiota of INS-GAS mice and may attenuate the severity of *H. pylori*-induced disease [97].

Limitations of Current Models and Alternatives to Investigate the Gastric Microbiota in Gastric Pathogenesis

Although great advances are being made in understanding the complex interplay between the microbiota and *H. pylori* in the development of gastric cancer in animal models, detailed molecular studies are still needed in well-defined human populations to examine differences in the microbiota of *H. pylori*-infected persons with and without gastric cancer [66]. Further, rodent models have several limitations including the finding that the phyla present in *H pylori*-infected human stomachs are not the same as those that predominate in a *H. pylori*-infected rodent stomach. Rodents are not naturally infected with *H. pylori* and need to be experimentally infected with rodent adapted strains. In addition, both the density and topography of *H. pylori* colonization in rodent stomachs does not precisely reflect that of humans [67]. Rodents possess a nonglandular forestomach, which is densely colonized by lactobacilli and can dramatically alter the composition of the rodent gastric microbiome, in contrast to what is present in the human stomach. In addition, some bacteria identified in the mouse stomach may be transient due to coprophagia, further confounding results [81].

The rhesus monkey (*Macaca mulatta*) is an exciting new model for studying the interactions between *H. pylori* and constituents of the gastric microbiota. Similar to humans, rhesus monkeys are naturally infected early in life with *H. pylori* strains which are indistinguishable from human strains. In addition, the anatomy of the rhesus monkey stomach is similar to humans, and multiple samples can be obtained over time by endoscopy [98]. A recent study in SPF rhesus monkeys using 454-pyrosequencing of the hyper-variable region of microbial 16S rRNA gene in

combination with high-throughput analysis of corpus and antral gastric biopsies reported that *Helicobacter* species dominate the gastric microbial community when present, although it should be noted that another 220 phylotypes were also detected. *Helicobacter* dominated the corpus to a larger degree than the antrum perhaps due to the lower pH found in the gastric corpus. However, infection with *H. pylori* was not found to significantly alter the relative abundance of other taxa [98].

Conclusions and Outlook

Gastric adenocarcinoma results in a high number of cancer-related deaths throughout the world and understanding the risk factors for this disease is crucial to identify individuals who are at highest risk for developing disease. Approximately half of the world's population is infected with *H. pylori*; however, 97–99 % of colonized persons will never develop gastric cancer. The risk of developing gastric cancer is multifactorial and recently, the role of the gastric microbiota as an important contributing factor in the progression towards gastric cancer has been identified. The role that the microbiota plays in obesity has been extensively studied and microbial genes can predict obesity with 90 % accuracy [99]. It is tempting to speculate that in the future, it may be possible to identify groups of bacterial taxa present in the stomach that are predictive of gastric disease outcome at specific stages along the Correa cascade. Indeed, it may also be possible to manipulate an individual's specific microbiota to proffer more favorable outcomes following infection with *H. pylori*. Detailed analyses of the human gastric microbiome still need to be conducted and it will be critical to carefully dissect causal versus effect changes. Importantly, delineation of the gastric microbiome is a rapidly evolving and exciting new area for research into the prevention and management of gastric disease.

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References

1. Ferlay J, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136(5):E359–86.
2. de Martel C, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. Lancet Oncol. 2012;13(6):607–15.
3. Parkin DM, et al. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55(2):74–108.
4. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society Award Lecture on cancer epidemiology and prevention. Cancer Res. 1992;52(24):6735–40.
5. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202–9.
6. Cristescu R, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21(5):449–56.

7. Fuchs CS, Mayer RJ. Gastric carcinoma. N Engl J Med. 1995;333(1):32–41.
8. Howson CP, Hiyama T, Wynder EL. The decline in gastric cancer: epidemiology of an unplanned triumph. Epidemiol Rev. 1986;8:1–27.
9. Blot WJ, et al. Rising incidence of adenocarcinoma of the esophagus and gastric cardia. JAMA. 1991;265(10):1287–9.
10. Pera M, et al. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. Gastroenterology. 1993;104(2):510–3.
11. Plummer M, et al. Global burden of gastric cancer attributable to pylori. Int J Cancer. 2014.
12. Uemura N, et al. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345(11):784–9.
13. Polk DB, Peek Jr RM. Helicobacter pylori: gastric cancer and beyond. Nat Rev Cancer. 2010;10(6):403–14.
14. Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. J Clin Invest. 2007;117(1):60–9.
15. Wroblewski LE, Peek Jr RM, Wilson KT. Helicobacter pylori and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev. 2010;23(4):713–39.
16. Linz B, et al. An African origin for the intimate association between humans and Helicobacter pylori. Nature. 2007;445(7130):915–8.
17. Peek Jr RM, Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol. 2006;208(2):233–48.
18. Wroblewski LE, Peek Jr RM. Helicobacter pylori in gastric carcinogenesis: mechanisms. Gastroenterol Clin North Am. 2013;42(2):285–98.
19. Odenbreit S, et al. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science. 2000;287(5457):1497–500.
20. Fischer W, et al. Systematic mutagenesis of the Helicobacter pylori cag pathogenicity island: essential genes for CagA translocation in host cells and induction of interleukin-8. Mol Microbiol. 2001;42(5):1337–48.
21. Kwok T, et al. Helicobacter exploits integrin for type IV secretion and kinase activation. Nature. 2007;449(7164):862–6.
22. Shaffer CL, et al. Helicobacter pylori exploits a unique repertoire of type IV secretion system components for pilus assembly at the bacteria-host cell interface. PLoS Pathog. 2011;7(9):e1002237.
23. Parsonnet J, et al. Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. Gut. 1997;40(3):297–301.
24. Huang JQ, et al. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. Gastroenterology. 2003;125(6):1636–44.
25. Hatakeyama M. Oncogenic mechanisms of the Helicobacter pylori CagA protein. Nat Rev Cancer. 2004;4(9):688–94.
26. Higashi H, et al. EPIYA motif is a membrane-targeting signal of Helicobacter pylori virulence factor CagA in mammalian cells. J Biol Chem. 2005;280(24):23130–7.
27. Naito M, et al. Influence of EPIYA-repeat polymorphism on the phosphorylation-dependent biological activity of Helicobacter pylori CagA. Gastroenterology. 2006;130(4):1181–90.
28. Basso D, et al. Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. Gastroenterology. 2008;135(1):91–9.
29. Argent RH, et al. Differences in Helicobacter pylori CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion. J Med Microbiol. 2008;57(Pt 9):1062–7.
30. Mimuro H, et al. Grb2 is a key mediator of helicobacter pylori CagA protein activities. Mol Cell. 2002;10(4):745–55.
31. Saadat I, et al. Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. Nature. 2007;447(7142):330–3.
32. Murata-Kamiya N, et al. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene. 2007;26(32):4617–26.

33. Churin Y, et al. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol. 2003;161(2):249–55.
34. Amieva MR, et al. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science. 2003;300(5624):1430–4.
35. Franco AT, et al. Activation of beta-catenin by carcinogenic Helicobacter pylori. Proc Natl Acad Sci U S A. 2005;102(30):10646–51.
36. Bagnoli F, et al. Helicobacter pylori CagA induces a transition from polarized to invasive phenotypes in MDCK cells. Proc Natl Acad Sci U S A. 2005;102(45):16339–44.
37. Suzuki M, et al. Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion. J Exp Med. 2005;202(9):1235–47.
38. Wroblewski LE, et al. Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. Gastroenterology. 2009;136(1):236–46.
39. Wroblewski LE, et al. Helicobacter pylori targets cancer-associated apical-junctional constituents in gastroids and gastric epithelial cells. Gut. 2015;64(5):720–30.
40. Cover TL, Blanke SR. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nat Rev Microbiol. 2005;3(4):320–32.
41. Boquet P, Ricci V. Intoxication strategy of Helicobacter pylori VacA toxin. Trends Microbiol. 2012;20(4):165–74.
42. Atherton JC, et al. Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem. 1995;270(30):17771–7.
43. Rhead JL, et al. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology. 2007;133(3):926–36.
44. Atherton JC, et al. Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. Gastroenterology. 1997;112(1):92–9.
45. Miehlke S, et al. The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. Int J Cancer. 2000;87(3):322–7.
46. Memon AA, et al. Vacuolating cytotoxin genotypes are strong markers of gastric cancer and duodenal ulcer-associated Helicobacter pylori strains: a matched case/control study. J Clin Microbiol. 2014;52(8):2984–9.
47. Winter JA, et al. A role for the vacuolating cytotoxin, VacA, in colonization and Helicobacter pylori-induced metaplasia in the stomach. J Infect Dis. 2014;210(6):954–63.
48. Backert S, Tegtmeyer N. The versatility of the Helicobacter pylori vacuolating cytotoxin VacA in signal transduction and molecular crosstalk. Toxins (Basel). 2010;2(1):69–92.
49. Barker N, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell. 2010;6(1):25–36.
50. Uehara T, et al. H. pylori infection is associated with DNA damage of Lgr5-positive epithelial stem cells in the stomach of patients with gastric cancer. Dig Dis Sci. 2013;58(1):140–9.
51. Tsugawa H, et al. Reactive oxygen species-induced autophagic degradation of Helicobacter pylori CagA is specifically suppressed in cancer stem-like cells. Cell Host Microbe. 2012;12(6):764–77.
52. El-Omar EM, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature. 2000;404(6776):398–402.
53. Figueiredo C, et al. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst. 2002;94(22):1680–7.
54. El-Omar EM, et al. Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. Gastroenterology. 2003;124(5):1193–201.
55. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. Gastric Cancer. 2007;10(2):75–83.
56. Epplein M, et al. Association of Helicobacter pylori infection and diet on the risk of gastric cancer: a case-control study in Hawaii. Cancer Causes Control. 2008;19(8):869–77.
57. Gonzalez CA, et al. Meat intake and risk of stomach and esophageal adenocarcinoma within the European Prospective Investigation Into Cancer and Nutrition (EPIC). J Natl Cancer Inst. 2006;98(5):345–54.

58. Gonzalez CA, et al. Fruit and vegetable intake and the risk of gastric adenocarcinoma: a reanalysis of the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study after a longer follow-up. Int J Cancer. 2012;131(12):2910–9.
59. Kim HJ, et al. Fresh and pickled vegetable consumption and gastric cancer in Japanese and Korean populations: a meta-analysis of observational studies. Cancer Sci. 2010;101(2):508–16.
60. Ren JS, et al. Pickled food and risk of gastric cancer—a systematic review and meta-analysis of English and Chinese literature. Cancer Epidemiol Biomarkers Prev. 2012;21(6):905–15.
61. Kim MK, et al. Prospective study of three major dietary patterns and risk of gastric cancer in Japan. Int J Cancer. 2004;110(3):435–42.
62. Lee SA, et al. Effect of diet and Helicobacter pylori infection to the risk of early gastric cancer. J Epidemiol. 2003;13(3):162–8.
63. Shikata K, et al. A prospective study of dietary salt intake and gastric cancer incidence in a defined Japanese population: the Hisayama study. Int J Cancer. 2006;119(1):196–201.
64. Noto JM, et al. Iron deficiency accelerates Helicobacter pylori-induced carcinogenesis in rodents and humans. J Clin Invest. 2013;123(1):479–92.
65. Ma JL, et al. Fifteen-year effects of Helicobacter pylori, garlic, and vitamin treatments on gastric cancer incidence and mortality. J Natl Cancer Inst. 2012;104(6):488–92.
66. Sheh A, Fox JG. The role of the gastrointestinal microbiome in Helicobacter pylori pathogenesis. Gut Microbes. 2013;4(6):505–31.
67. Abreu MT, Peek Jr RM. Gastrointestinal malignancy and the microbiome. Gastroenterology. 2014;146(6):1534–46. e3.
68. Bik EM, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci U S A. 2006;103(3):732–7.
69. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet. 2012;13(4):260–70.
70. Andersson AF, et al. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One. 2008;3(7):e2836.
71. Maldonado-Contreras A, et al. Structure of the human gastric bacterial community in relation to Helicobacter pylori status. ISME J. 2011;5(4):574–9.
72. Dicksved J, et al. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. J Med Microbiol. 2009;58(Pt 4):509–16.
73. Eun CS, et al. Differences in gastric mucosal microbiota profiling in patients with chronic gastritis, intestinal metaplasia, and gastric cancer using pyrosequencing methods. Helicobacter. 2014;19(6):407–16.
74. Peek Jr RM, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer. 2002;2(1):28–37.
75. Watanabe T, et al. Helicobacter pylori infection induces gastric cancer in mongolian gerbils [see comments]. Gastroenterology. 1998;115(3):642–8.
76. Honda S, et al. Development of Helicobacter pylori-induced gastric carcinoma in Mongolian gerbils. Cancer Res. 1998;58(19):4255–9.
77. Ogura K, et al. Virulence factors of Helicobacter pylori responsible for gastric diseases in Mongolian gerbil. J Exp Med. 2000;192(11):1601–10.
78. Peek RM, et al. Helicobacter pylori alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. Gastroenterology. 2000;118(1):48–59.
79. Israel DA, et al. Helicobacter pylori strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. J Clin Invest. 2001;107(5):611–20.
80. Franco AT, et al. Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors. Cancer Res. 2008;68(2):379–87.
81. Fox J, Sheh A. The role of the gastrointestinal microbiome in Helicobacter pylori pathogenesis. Gut Microbes. 2013;4(6):505–31.
82. Yang I, Nell S, Suerbaum S. Survival in hostile territory: the microbiota of the stomach. FEMS Microbiol Rev. 2013;37(5):736–61.
83. Sun YQ, et al. Profiling and identification of eubacteria in the stomach of Mongolian gerbils with and without Helicobacter pylori infection. Helicobacter. 2003;8(2):149–57.

84. Osaki T, et al. Comparative analysis of gastric bacterial microbiota in Mongolian gerbils after long-term infection with Helicobacter pylori. Microb Pathog. 2012;53(1):12–8.
85. Zaman C, et al. Analysis of the microbial ecology between Helicobacter pylori and the gastric microbiota of Mongolian gerbils. J Med Microbiol. 2014;63(Pt 1):129–37.
86. Rolig AS, et al. The degree of Helicobacter pylori-triggered inflammation is manipulated by preinfection host microbiota. Infect Immun. 2013;81(5):1382–9.
87. Zavros Y, et al. Gastritis and hypergastrinemia due to Acinetobacter lwoffii in mice. Infect Immun. 2002;70(5):2630–9.
88. Sigal M, et al. Helicobacter pylori activates and expands Lgr5 stem cells through direct colonization of the gastric glands. Gastroenterology. 2015;148(7):1392–404.e21.
89. Aebischer T, et al. Vaccination prevents Helicobacter pylori-induced alterations of the gastric flora in mice. FEMS Immunol Med Microbiol. 2006;46(2):221–9.
90. Tan MP, et al. Chronic Helicobacter pylori infection does not significantly alter the microbiota of the murine stomach. Appl Environ Microbiol. 2007;73(3):1010–3.
91. Lofgren JL, et al. Lack of commensal flora in Helicobacter pylori-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. Gastroenterology. 2011;140(1):210–20.
92. Thomson MJ, et al. Gastric Helicobacter infection induces iron deficiency in the INS-GAS mouse. PLoS One. 2012;7(11):e50194.
93. Wang J, et al. Helicobacter pylori modulates lymphoepithelial cell interactions leading to epithelial cell damage through Fas/Fas ligand interactions. Infect Immun. 2000;68(7):4303–11.
94. Lertpiriyapong K, et al. Gastric colonisation with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the Helicobacter pylori INS-GAS mouse model of gastric carcinogenesis. Gut. 2014;63(1):54–63.
95. Ge Z, et al. Coinfection with enterohepatic Helicobacter species can ameliorate or promote Helicobacter pylori-induced gastric pathology in C57BL/6 mice. Infect Immun. 2011;79(10):3861–71.
96. Lemke LB, et al. Concurrent Helicobacter bilis infection in C57BL/6 mice attenuates proinflammatory H. pylori-induced gastric pathology. Infect Immun. 2009;77(5):2147–58.
97. Whary MT, et al. Helminth co-infection in Helicobacter pylori infected INS-GAS mice attenuates gastric premalignant lesions of epithelial dysplasia and glandular atrophy and preserves colonization resistance of the stomach to lower bowel microbiota. Microbes Infect. 2014;16(4):345–55.
98. Martin ME, et al. The impact of Helicobacter pylori infection on the gastric microbiota of the rhesus macaque. PLoS One. 2013;8(10):e76375.
99. DeWeerdt S. Microbiome: a complicated relationship status. Nature. 2014;508(7496):S61–3.

Chapter 20
Helicobacter pylori and Gastric Cancer: Timing and Impact of Preventive Measures

Marino Venerito, Riccardo Vasapolli, and Peter Malfertheiner

Gastric Cancer: Classification

GC is classified anatomically as *proximal* (cardia) and *distal* (non-cardia). According to the Lauren classification gastric cancer can be subdivided in two distinct pathological entities: the *intestinal type* and the *diffuse type* [1]. Sporadic non-cardia GC of both intestinal and diffuse type is commonly associated with *H. pylori* infection, whereas the association of this infection with cardia GC is less well defined. In the present chapter we give a special emphasis to non-cardia GC because of its close association with *H. pylori* infection.

Gastric Cancer: Epidemiological Aspects

H. pylori infection is the main risk factor involved in gastric carcinogenesis and subsequently GC incidence tends to mirror the prevalence rate of *H. pylori* infection [2]. GC represents the third leading cause of cancer related death in the world [3]. Despite the fact that a continuing decrease in the incidence of gastric carcinoma has been observed during the last decades, this malignancy still represents an important health burden in all almost populations and causes more than 720,000 deaths per year globally. There is a substantial geographic variation in GC incidence, with the highest rates reported in Eastern Asia (age-standardized incidence 35.4 per 100,000 in men, 13.8 per 100,000 in women). High incidence and mortality rates are also observed in both sexes in Central and Eastern Europe and in Central and South America [4]. Men have approximately twice the risk of developing stomach cancer, compared to women [5]. Outlook for patients with gastric cancer remains poor, principally due to the advanced stage of the disease frequently observed at first diagnosis. The mortality rates for gastric adenocarcinoma are similar in most Western countries with relative 5-year survival rates generally lower than 30 % [6].

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In contrast, the survival data from Eastern countries including Korea and Japan are more favorable with overall 5-year survival rates up to 60 %, likely following the adopted mass screening programs that facilitate earlier diagnosis of the disease [7].

H. pylori as Principal Trigger of Gastric Carcinogenesis

Gastric carcinogenesis is a complex multistep process in which different etiologic factors are implicated. Numerous lifestyle and dietetic habits have been indicated as a risk factor for non-cardia GC. High intake of salt as well as traditional salt-preserved foods, smoked or dried meat, and fish, combined with a decreased intake of fibers, fresh fruit and vegetables significantly increase the risk of non-cardia GC occurrence [8]. Several studies have highlighted the possible impact of exposure to N-nitroso compounds and/or polycyclic aromatic amines on non-cardia GC development [9]. Moreover, low socioeconomic status, low level of physical activity, obesity, radiation exposure, and cigarette smoking have also been associated with an increased risk of non-cardia GC [10–12]. The strongest confirmed and widely recognized risk factor for GC, however, is infection with *H. pylori* [13]. A causal relationship between the chronic inflammatory process of the gastric mucosa triggered by this bacterium and subsequent gastric carcinogenesis has been consolidated in the past years. Indeed, in 1994 the International Agency for Research on Cancer (IARC), part of the World Health Organization (WHO), formally classified *H. pylori* as a class I "definite carcinogen" [14]. The more recent WHO contribution by the IARC published in 2010 confirmed that chronic infection with *H. pylori* is carcinogenic to humans. This confirmation is based on multiple lines of evidence pointing to a central role for the chronic gastric inflammatory response and resulting oxidative stress in *H. pylori*-associated gastric carcinogenesis [15]. The development of non-cardia intestinal type gastric cancer is a multistep process triggered by *H. pylori* infection (Fig. 20.1). This multistep model, which was in large part developed by Correa and colleagues, is based on a temporal sequence of precancerous changes that eventually leads to the development of gastric cancer [16]. *H. pylori*-induced gastric inflammation represents a common feature of the initiation and progression to intestinal type GC. In a subset of patients, the chronic inflammatory process leads to the loss of gastric glandular tissue (atrophy) and replacement of the normal gastric lineages with metaplastic cells (intestinal metaplasia) [17, 18]. Chronic atrophic gastritis is a precancerous condition occurring in *H. pylori*-infected subjects at an annual incidence rate of 1–3 % [19]. Dysplasia, early GC and advanced GC may follow. Further concomitant variables such as host genetic predisposition and infection with *H. pylori* strains with specific virulence factors (mainly CagA and VacA) contribute to mucosal injury increasing the individual risk of GC development [20].

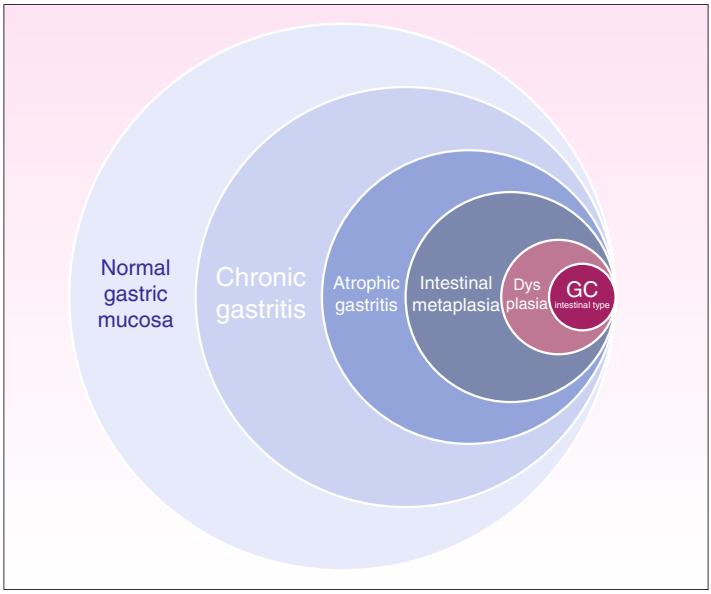

Fig. 20.1 Russian doll model of the Correa cascade. The Correa cascade is a multistep model based on a temporal sequence of precancerous changes that eventually lead to the development of gastric cancer on the basis of *Helicobacter pylori*-driven chronic active gastritis. The latter occurs within a timeframe of months while the development of dysplasia takes years to decades. Proportions not drawn to scale

Gastric Cancer: Prevention Strategies

Population-based strategies for primary prevention of GC depend on the local GC prevalence and on their cost-effectiveness. In areas with high GC prevalence, *H. pylori* eradication is the most effective strategy for preventing GC. A recent meta-analysis including 6497 subjects from six randomized controlled clinical trials showed a reduced incidence of GC in *H. pylori* infected individuals who received eradication therapy compared with controls assigned to placebo or no treatment after a follow-up of 4–14.7 years (OR: 0.66; 95 % CI: 0.46–0.95) [21]. Similar results have been reported in another meta-analysis from Chen et al. including ten studies from eight randomized clinical studies for a total of 7955 participants. The risk of GC development was lower in the *H. pylori* eradicated group compared with controls, with a pooled RR of 0.64 (95 % CI, 0.48–0.85). However, in the subgroup analysis of patients with intestinal metaplasia or dysplasia, this difference was not observed (RR = 0.88; 95 % CI, 0.59–1.31) [22]. Thus, the beneficial effect of *H. pylori* eradication in terms of GC prevention is restricted to *H. pylori*-infected patients without advanced precancerous conditions at baseline [23].

H. pylori eradication has been evaluated also for prevention of metachronous lesions in patients who received endoscopic removal of an early GC. It has been estimated, namely, that after endoscopic resection of early GC *H. pylori*-infected

patients who do not receive prophylactic eradication have an annual risk of developing metachronous GC between 3 and 4 % per year (e.g., 3000–4000/100,000 per year) [24]. To date, there are only two randomized controlled studies evaluating the role of *H. pylori* eradication for preventing metachronous lesions after endoscopic removal of an early GC. In a multicentre prospective randomized study including 544 Japanese patients with a follow-up period of 3 years after endoscopic removal of an early GC, metachronous GC developed in 9/272 (3.3 %) patients with *H. pylori* eradication and in 24/272 (8.8 %) controls [25]. The significant reduction of the incidence of metachronous GC (OR = 0.35, 95 % CI: 0.16–0.78; $p = 0.009$) was consistent with a role of *H. pylori* eradication in delaying the onset of new cancers in the same stomach. In contrast, another prospective, randomized, open-label trial including 901 consecutive Korean patients, eradication of *H. pylori* did not found any reduction in the incidence of metachronous gastric carcinoma after endoscopic resection of gastric dysplasia or early GC [26]. In this study, patients underwent endoscopic follow-up at 3, 6, and 12 months after treatment and yearly thereafter. During a median follow-up period of 3 years, ten patients who received *H. pylori* eradication and 17 controls developed metachronous carcinoma; this difference was not significant ($p = 0.15$). The incidence of metachronous carcinoma did not differ significantly at 1, 2, 3, and 4 years after administration of the therapy between the two groups. There were no significant differences in the development of metachronous carcinoma among patients who were positive ($n = 16$) or negative ($n = 11$) for *H. pylori* infection ($p = 0.32$). Baseline characteristics of the patients included in the study as well as study design may account for the contrasting results of the two prospective trials.

The importance of the "test-and-treat" strategy to reach a mass eradication of *H. pylori* has been recently discussed in a Working Group meeting of the IARC. The Working Group considered that the implementation of a population-based screening would certainly have a relevant impact not only on GC incidence but also on other *H. pylori*-related diseases, such as peptic ulcer disease, MALT lymphoma, and dyspepsia. This approach would be in most cases cost-effective, in particular in high-risk areas, nevertheless resource availability, health priorities, feasibility, logistics, and possible adverse consequences should be accurately evaluated during the implementation [27]. To date nation-wide screening programs for *H. pylori* eradication and early diagnosis of GC have been implemented in Japan and Korea [7, 28]. Other case-control and cohort studies are ongoing in different high-risk region in China, Taiwan, and Europe and new insights to determine the best management strategies for GC prevention are expected in the following years [29–31].

H. pylori and Other Gastrointestinal Malignancies

A possible role of *H. pylori* in triggering some extragastric gastrointestinal neoplasms has been increasingly investigated over the past years. Gastric colonization with *H. pylori* has been associated with different malignancies including

Table 20.1 Most recent studies on the association between *H. pylori* and extragastric malignancies

Reference	Type of study	Type of malignancy	Population/group	Odds ratio; 95 % confidence interval
Nie et al. [30]	Meta-analysis	Esophageal adenocarcinoma (EA)	Overall population (both CagA+ and CagA- strains)	0.57 (95 % CI, 0.44–0.73)
		Esophageal squamous cell carcinoma (ESCC)	Non-Asian population (CagA+ strains)	1.16 (95 % CI, 0.83–1.60)
			Asian population (CagA+ strains)	0.97 (95 % CI, 0.79–1.19)
			Non-Asian population (CagA- strains)	1.41 (95 % CI, 1.02–1.94)
			Asian population (CagA- strains)	0.74 (95 % CI, 0.57–0.97)
Murphy et al. [35]	Prospective study	Biliary tract cancer	Finnish population	2.63 (95 % CI: 1.08–6.37)
		Hepatocellular cancer		1.91 (95 % CI: 0.69–5.29)
Schulte et al. [37]	Meta-analysis	Pancreatic cancer	*H. pylori* CagA+ strains	0.78 (95 % CI: 0.67–0.91)
			H. pylori CagA- strains	1.30 (95 % CI: 1.02–1.65)
Sonnenberg et al. [38]	Retrospective study	Colon adenomatous polyps	American population	1.52 (95 % CI, 1.46–1.57)
		Colon adenomas with high-grade IEN		1.97 (95 % CI, 1.82–2.14)
		Colorectal cancer		2.35 (95 % CI, 1.98–2.80)

esophageal, pancreatic, hepatocellular, and colon cancer. However there is some discrepancy in the results reported in the literature and a causative role of the bacterium in the process of carcinogenesis outside the stomach has not yet been demonstrated. A description of most recent studies and meta-analysis present in the literature that reported an association between *H. pylori* and extragastric malignancies is presented in Table 20.1.

Esophageal Cancer

An inverse relationship between *H. pylori* infection and esophageal adenocarcinoma (EA) had been reported since 1997 [32]. In subsequent studies the effect of specific strains of *H. pylori* on esophageal cancer was examined and it was suggested that CagA-positive strains of *H. pylori* are associated with a significantly

reduced occurrence of EA [33]. In a recent meta-analysis from Nie et al. including 28 selected studies this significant inverse relationship between *H. pylori* infection and esophageal adenocarcinoma (EAC) was confirmed (pooled OR, 0.57; 95 % CI, 0.44–0.73) [34]. No significant association with esophageal squamous cell carcinoma (ESCC) in the overall population was described. After stratification for study location, however, a slightly increased risk for ESCC in non-Asian patients infected with CagA-positive strains was observed (OR 1.41; 95 % CI, 1.02–1.94), whereas Asian patients infected with CagA-positive *H. pylori* had a lower risk for ESCC (OR 0.74; 95 % CI, 0.57–0.97). Also the influence of *H. pylori* on the development of esophageal preneoplastic conditions seems to be population-dependent. In a single-center case-control study on 1952 African Americans and Non-Hispanic Whites the prevalence of Barrett's esophagus (BE) was related to various sociodemographic and clinical factors [35]. The prevalence of BE among Non-Hispanic white patients with *H. pylori* infection was significantly decreased in comparison with *H. pylori* negative controls. In the African American group no significant relationship between *H. pylori* and BE was found. In a similar manner, presence of esophageal dysplasia was inversely related with *H. pylori* infection in a cohort of 151 Japanese patients with BE [36]. The pathophysiologic mechanisms underlying the supposed protective role of *H. pylori* against esophageal adenocarcinoma have not been fully elucidated. The most plausible hypothesis is that *H. pylori*-related hypochlorhydria (secondarily) attenuates the caustic impact of gastro-oesophageal reflux and, as a result, decreases the development of preneoplastic conditions such as Barrett's oesophagus [37].

Malignancies of the Hepatobiliopancreatic Tract

The presence of different *Helicobacter* species (spp.) in bile, gallstones, biliary, and hepatic tissues has been recently reported, so that an involvement of these bacteria in the pathogenesis of hepatobiliary neoplasms has been speculated [38]. Analyzing the seropositivity to multiple *H. pylori* antigens in blood samples collected in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) Murphy et al. investigated the association between *H. pylori* infection and occurrence of hepatobiliary cancers in a total population of 29,133 Finnish male smokers, 50–69 years old, prospectively recruited between 1985 and 1988 [39]. During a follow-up of 22 years 64 biliary cancers and 122 liver cancers have been diagnosed. The seroprevalence of *H. pylori* infection at baseline was 88 % among controls, but higher among those who subsequently developed hepatobiliary cancer: 100 % for gallbladder cancer, 97 % of extrahepatic bile duct cancer, 91 % of ampulla of Vater cancer, 96 % of intrahepatic bile duct cancer, and 94 % of hepatocellular carcinoma. Seropositivity to *H. pylori* antigens was associated with an increased risk of biliary tract cancers in general (multivariate adjusted OR 2.63; 95 % CI: 1.08–6.37) and biliary cancer (multivariate adjusted OR: 5.47; 95 % CI: 1.17–25.65), but not liver cancer (multivariate adjusted OR: 1.91; 95 % CI: 0.69–5.29). Furthermore, in a clinicopathological study

from Hassan et al. detection of *H. pylori* in the gallbladder tissue from patients who underwent cholecystectomy for chronic cholecystitis was related to more severe histopathological lesions (mucosal hyperplasia, metaplasia/dysplasia, and lymphoid infiltration) in comparison with the *H. pylori*-negative group [40].

Studies with regards to the possible influence of *H. pylori* on the pathogenesis of pancreatic cancer have shown inconsistent results. A statistically significant strain-specific association between *H. pylori* and pancreatic cancer risk was recently observed in a meta-analysis of ten studies including 580 patients and 626 controls [41]. While no significant link between *H. pylori* seropositivity and pancreatic cancer risk in the overall population was observed (OR 1.13; 95 % CI 0.86–1.50), the authors report a CagA status-dependent relationship in the sub-analysis. Patients infected with CagA-positive strains were less likely to have pancreatic cancer (OR: 0.78; 95 % CI: 0.67–0.91), whereas a slightly increased rate of pancreatic cancer was observed in patients with CagA-negative strains (OR: 1.30; 95 % CI: 1.02–1.65).

Colorectal Neoplasms

Several studies have suggested that *H. pylori* positive patients also have a moderately increased risk of developing colorectal malignancies. A strong association between *H. pylori*-related gastritis and the development of different colonic neoplasm (including hyperplastic polyps, adenomatous polyps, and adenocarcinomas) was observed in a large retrospective study analyzing 156,000 subjects who underwent colonoscopy and esophagogastroduodenoscopy [42]. In support of these findings, a higher prevalence of colorectal lesions was observed in *H. pylori*-infected patients with respect to *H. pylori* negative controls (43 % vs. 34 %; OR: 1.5; 95 % CI: 1.2–1.9) among 1256 African Americans who underwent esophagogastroduodenoscopy (EGD) and colonoscopy on the same day [43]. However, no association between *H. pylori* infection and colorectal adenomas has been reported in similar analyses of Hispanic and East Asian cohorts [44, 45], indicating that possibly this relationship is population-dependent. Further work on this important topic remains to be done.

Conclusion

GC is the third leading cause of cancer related death worldwide at present. *H. pylori*-infection represents the major risk factor for the development of sporadic non-cardia gastric cancer (GC) of both intestinal and diffuse type. The most efficacious to reduce GC-related mortality remains prevention. Mass eradication of *H. pylori* infection has been tested in countries with high GC prevalence and has shown that the beneficial effect with regards to prevention of GC is best in *H. pylori*-infected patients without precancerous lesions. A target age for this test-and-treat strategy has

not been defined as yet, but treatment should precede the appearance of precancerous lesions: the earlier the treatment, the higher the expected benefit.

With respect to extragastric gastrointestinal malignancies, growing epidemiological data has shown an inverse association of *H. pylori*-infection with esophageal adenocarcinoma and squamous cell carcinoma, and a positive association with pancreatic, biliary tract, and colorectal cancers although mechanistic roles of the bacterium in carcinogenesis outside of the stomach has not been demonstrated. This is clearly an area for future research.

References

1. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histoclinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
2. Fock KM, Ang TL. Epidemiology of Helicobacter pylori infection and gastric cancer in Asia. J Gastroenterol Hepatol. 2010;25:479–86.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.
4. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer; 2013. http://globocan.iarc.fr.
5. Carneiro F. Stomach cancer. In: Steward BW, Wild CP, editors. World cancer report 2014. Lyon: International Agency for Research on Cancer; 2014. p. 383–91.
6. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893–917.
7. Asaka M, Kato M, Sakamoto N. Roadmap to eliminate gastric cancer with Helicobacter pylori eradication and consecutive surveillance in Japan. J Gastroenterol. 2014;49:1–8.
8. Tsugane S, Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. Gastric Cancer. 2007;10(2):75–83.
9. Jakszyn P, Bingham S, Pera G, Riboli E, Gonzalez CA, et al. Endogenous versus exogenous exposure to N-nitroso compounds and gastric cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC-EURGAST) study. Carcinogenesis. 2006;27(7):1497–501.
10. O'Doherty MG, Freedman ND, Hollenbeck AR, et al. A prospective cohort study of obesity and risk of oesophageal and gastric adenocarcinoma in the NIH-AARP Diet and Health Study. Gut. 2012;61:1261–8.
11. Ladeiras-Lopes R, Pereira AK, Nogueira A, et al. Smoking and gastric cancer: systematic review and meta-analysis of cohort studies. Cancer Causes Control. 2008;19:689–701.
12. Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev. 2014;23:700–13.
13. Malfertheiner P, Link A, Selgrad M. Helicobacter pylori: perspectives and time trends. Nat Rev Gastroenterol Hepatol. 2014;11(10):628–38.
14. IARC Working Group. IARC monographs on the evaluation of carcinogenic risk to humans. Schistosomes, liver flukes and *Helicobacter Pylor*, vol. 61. IARC: Lyon, France; 1994.
15. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Biological agents. Volume 100 B. A review of human carcinogens. IARC Monogr Eval Carcinog Risks Hum. 2012;100(Pt B):1–441.

16. Correa P, Haenszel W, Cuello C, Tannenbaum S, Archer M. A model for gastric cancer epidemiology. Lancet. 1975;2:58–60.
17. El-Omar EM, Oien K, El Nujumi A, Gillen D, Wirz A, Dahill S, Williams C, Ardill JE, McColl KE. *Helicobacter pylori* infection and chronic gastric acid hyposecretion. Gastroenterology. 1997;113:15–24.
18. Uemura N, et al. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
19. Vannella L, Lahner E, Annibale B. Risk for gastric neoplasias in patients with chronic atrophic gastritis: a critical reappraisal. World J Gastroenterol. 2012;18(12):1279–85.
20. Huang JQ, Zheng GF, Sumanac K, et al. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. Gastroenterology. 2003;125:1636.
21. Ford AC, Forman D, Hunt RH, Yuan Y, Moayyedi P. Helicobacter pylori eradication therapy to prevent gastric cancer in healthy asymptomatic infected individuals: systematic review and meta-analysis of randomised controlled trials. BMJ. 2104;348:3174.
22. Chen HN, Wang Z, Li X, Zhou ZG. Helicobacter pylori eradication cannot reduce the risk of gastric cancer in patients with intestinal metaplasia and dysplasia: evidence from a meta-analysis. Gastric Cancer. 2015;22. Epub ahead of print.
23. Venerito M, Malfertheiner P. Preneoplastic conditions in the stomach: always a point of no return? Dig Dis. 2105;33(1):5–10.
24. Shiotani A, Haruma K, Graham DY. Metachronous gastric cancer after successful Helicobacter pylori eradication. World J Gastroenterol. 2014;20(33):11552–9.
25. Fukase K, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, Terao S, Amagai K, Hayashi S, Asaka M. Effect of eradication of Helicobacter pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. Lancet. 2008;372:392–7.
26. Choi J, Kim SG, Yoon H, Im JP, Kim JS, Kim WH, Jung HC. Eradication of Helicobacter pylori after endoscopic resection of gastric tumors does not reduce incidence of metachronous gastric carcinoma. Clin Gastroenterol Hepatol. 2014;12:793–800.
27. Herrero R, Park JY, Forman D. The fight against gastric cancer—the IARC Working Group report. Best Pract Res Clin Gastroenterol. 2014;28(6):1107–14.
28. Cho E, Kang MH, Choi KS, Suh M, Jun JK, Park EC. Cost-effectiveness outcomes of the national gastric cancer screening program in South Korea. Asian Pac J Cancer Prev. 2013;14(4):2533–40.
29. Lu YL, Lui LY, Wu Q, Lui WD, Li SJ, Cao CQ, et al. Comparison of two cancer screening schemes in a high-risk population. Chin J Oncol. 2012;35:394e7.
30. Malfertheiner P, Selgrad M. Gastric cancer screening in conjunction with colorectal cancer screening in Europe (GACSE). Clin Stud Protoc Version 1. 2013.
31. Leja M, Herrero R, Park JY, Plummer M. Multicentric randomized study of Helicobacter pylori eradication and pepsinogen testing for prevention of gastric cancer mortality (gastric cancer prevention study by predicting atrophic gastritis; GISTAR). In: Helicobacter pylori eradication as a strategy for preventing gastric cancer. IARC Helicobacter pylori Working Group. Lyon, France: International Agency for Research on Cancer (IARC Working Group Reports, No. 8); 2014. Ch 4.2., p. 1474–1153.
32. Newton M, Bryan R, Burnham WR, Kamm MA. Evaluation of Helicobacter pylori in reflux oesophagitis and Barrett's oesophagus. Gut. 1997;40:9–13.
33. Chow WH, Blaser MJ, Blot WJ, Gammon MD, et al. An inverse relation between cagA+ strains of Helicobacter pylori infection and risk of esophageal and gastric cardia adenocarcinoma. Cancer Res. 1998;58(4):588–90.
34. Nie S, Chen T, Yang X, Huai P, Lu M. Association of Helicobacter pylori infection with esophageal adenocarcinoma and squamous cell carcinoma: a meta-analysis. Dis Esophagus. 2014;27(7):645–53.
35. Nguyen TH, Thrift AP, Ramsey D, Green L, Shaib YH, Graham DY, El-Serag HB. Risk factors for Barrett's esophagus compared between African Americans and non-Hispanic Whites. Am J Gastroenterol. 2014;109(12):1870–80.

36. Fujita M, Nakamura Y, Kasashima S, Furukawa M, Misaka R, Nagahara H. Risk factors associated with Barrett's epithelial dysplasia. World J Gastroenterol. 2014;20(15):4353–61.
37. Rokkas T, Pistiolas D, Sechopoulos P, Robotis I, Margantinis G. Relationship between Helicobacter pylori infection and esophageal neoplasia: a meta-analysis. Clin Gastroenterol Hepatol. 2007;5(12):1413–7.
38. Lee JW, Lee DH, Lee JI, Jeong S, Song SY, et al. Identification of Helicobacter pylori in gallstone, bile, and other hepatobiliary tissues of patients with cholecystitis. Gut Liver. 2010;4(1):60–7.
39. Murphy G, Michel A, Taylor PR, Albanes D, Weinstein SJ, Virtamo J, Parisi D, Snyder K, Butt J, McGlynn KA, Koshiol J, Pawlita M, Lai GY, Abnet CC, Dawsey SM, Freedman ND. Association of seropositivity to Helicobacter species and biliary tract cancer in the ATBC study. Hepatology. 2014;60(6):1963–71.
40. Hassan EH, Gerges SS, El-Atrebi KA, El-Bassyouni HT. The role of H. pylori infection in gall bladder cancer: clinicopathological study. Tumour Biol. [Epub ahead of print]. Accessed 16 Apr 2015.
41. Schulte A, Pandeya N, Fawcett J, Fritschi L, Risch HA, Webb PM, Whiteman DC, Neale RE. Association between Helicobacter pylori and pancreatic cancer risk: a meta-analysis. Cancer Causes Control. 2015;26(7):1027–35.
42. Sonnenberg A, Genta RM. Helicobacter pylori is a risk factor for colonic neoplasms. Am J Gastroenterol. 2013;108(2):208–15.
43. Brim H, Zahaf M, Laiyemo AO, Nouraie M, Pérez-Pérez GI, Smoot DT, Lee E, Razjouyan H, Ashktorab H. Gastric Helicobacter pylori infection associates with an increased risk of colorectal polyps in African Americans. BMC Cancer. 2014;14:296.
44. Patel S, Lipka S, Shen H, Barnowsky A, Silpe J, Krishnamachari B, et al. The association of H. pylori and colorectal adenoma: does it exist in the US Hispanic population? J Gastrointest Oncol. 2014;5(6):463–8.
45. Guo Y, Li HY. Association between Helicobacter pylori infection and colorectal neoplasm risk: a meta-analysis based on East Asian population. J Cancer Res Ther. 2014;10(Suppl):263–6.

Chapter 21
Genomics Study of Gastric Cancer and Its Molecular Subtypes

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Gastric cancer encompasses several distinct morphological and molecular subtypes, and is associated with widely different incidence and etiological agents in different ethnic groups. Previous genetic studies have identified a few molecular subtypes with distinct cancer initiating events. Recently, large-scale genomic studies have become possible due to the availability of next-generation sequencing technology and various array-based assessment methods. Integration of these diverse molecular parameters has led to an unparalleled opportunity to uncover the molecular portrait of gastric cancer and reveal the complex genetic and epigenetic perturbations accumulating in the gastric cancer genome that underscore the molecular complexity of the process of carcinogenesis. One of the key problems arising from these genomic studies is the difficulty in distinguishing driver from passenger mutations. Furthermore, our ability and speed in identifying mutations far exceed our ability and speed to functionally characterize each of these alterations. Thus, the advances in these genomic studies mark the beginning of a new era in which further bioinformatics and high-throughput functional genomic screening technology will be needed to fully harness their maximum potential. Recent advances in genome editing technology and organoid cultures are some of the opportunities lying ahead for moving forward with these studies and the potential to link genomic alterations with therapy.

Molecular and Morphological Diversity of Gastric cancer

Gastric cancer can be distinguished into the intestinal and diffuse type based on Lauren's classification. This is purely based on morphological assessment of glandular formation in the former versus early loss in cell-to-cell adhesion, leading to widely infiltrative growth usually accompanied by an excessive amount of desmoplastic stroma in the later. In terms of molecular pathway, two distinct molecular subtypes of gastric cancer have well-documented initiating driver events. The MSI

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subtype is mostly due to promoter methylation leading to transcriptional silencing of the DNA mismatch repair gene MLH1, resulting in a form of genomic instability known as microsatellite instability [1]. In a minor proportion of cases, MSI gastric cancer can also result from germline mutation of the DNA MMR gene, as part of Lynch Syndrome. Microsatellite instability leads to frequent frameshift mutations in repeat tracts of DNA, causing inactivating mutations of key tumor suppressors, or frequent missense-activating mutations in oncogenes [2, 3]. The second distinct molecular subtype is due to infection by the Epstein-Barr virus [4, 5], where early entry of the virus into a single host cell leads to clonal expansion and cancer development. In the latent state of infection, the virus is always in clonal episomal form [6]. Each of these molecular subtypes has specific clinicopathological associations. For example, MSI gastric cancers have a predilection for antral location and are associated with *Helicobacter pylori* infection, as well as intestinal metaplasia, and a better prognosis [2, 7]. The EBV-associated GCs, by contrast, have a predilection for gastric body and cardia, and absence of intestinal metaplasia. There is accompanying intense reactive lymphoid infiltration in both types, with the EBV-associated type occasionally displaying the lymphoepithelioma-like carcinoma (LELC) phenotype, closely mimicking its nasopharyngeal counterpart. Interestingly, both molecular subtypes have been shown to display the CpG island methylator phenotype (CIMP) [8–10]. Also, both molecular subtypes are more commonly associated with intestinal-type gastric cancers.

Helicobacter pylori infection in the stomach triggers a cascade of events including chronic gastritis, intestinal metaplasia, followed by progression to cancer. Thus, intestinal metaplasia is considered a pre-neoplastic process that marks an increased risk for gastric cancer development. Our previous gene expression profiling study of intestinal metaplasia and gastric cancer showed that during the metaplastic process, a transcriptional program characterized by induction of the CDX1 and CDX2 along with genes of intestinal differentiation such as Villin1, LI-cadherin, and TFF3, are expressed in intestinal metaplasia, as well as a subset of gastric cancers that display a marked degree of intestinal metaplasia at the tumor edge, supporting the notion that gastric cancer with this transcriptional program may have progressed from intestinal metaplasia [11]. Interestingly, there is no association of the presence or absence of this transcriptional program with the Lauren's classification, which merely described the presence or absence of glandular formation, irrespective of the lineage of differentiation of the glands to be gastric or intestinal in origin.

Prior to the availability of large-scale genomic studies, only a few driver mutations were known in gastric cancer, some with pathway-specific affinity or exclusivities. TP53 is the most frequently mutated driver gene, along with CDH1, the latter is predominantly mutated in diffuse-type gastric cancers [12]. Mutation of the WNT signaling pathway genes, including APC and beta-catenin, has been described mostly in intestinal-type gastric cancers, albeit at a substantially lower frequency compared to colorectal cancer [13–15]. Aside from these, other driver genes are reported to be mutated mostly in only a small proportion of gastric cancers. Some of these morphological or molecular subgroups have been shown to have prognostic significance. For example, diffuse-type gastric cancers are associated with poor

prognosis [16], whereas the MSI or EBV gastric cancers are associated with better prognosis [17, 18].

Whole-Exome and Whole-Genome Sequencing Study of Gastric Cancer

Advances in next-generation sequencing have facilitated several small-scale studies, followed by a few large-scale comprehensive sequencing studies of gastric cancer on a whole-exome or whole-genome scale, covering diverse populations including Hong Kong, China, and Asian and Western countries [19–26]. The emerging picture shows a few consistent patterns of pathway-specific driver gene alterations commonly seen across different studies, as well as unique driver genes reported only in specific studies. A common problem that emerges with these genome-scale sequencing studies is the difficulty in distinguishing driver from passenger mutations. Earlier studies reporting gene mutations as drivers are mostly based on their high recurrent mutation rates. However, several confounding factors are immediately obvious. Firstly, large genes are more likely to have mutations than small genes, as evidenced by the fact that one of the most frequently observed mutated genes is Titin, the largest known gene in humans. Secondly, passenger mutations are substantially more common in hypermutated tumors (i.e. those with MSI or POLE mutation), simply because of the inherent genetic instability. Thirdly, mutation rates are substantially higher in genes that are not transcribed compared with genes that are highly transcribed. Fourthly, mutation rates are different in different nucleotide and context, e.g. mutations are more common in CpG dinucleotide because of spontaneous deamination leading to C>T mutation. Subsequently, large-scale sequencing of diverse cancer types has led to identification of more factors that could lead to an elevated mutation rate in different parts of the genome [27]. Mutation rates vary with regard to closed chromatin versus open chromatin, DNA replication origin, etc. Based on this information, a number of driver gene algorithms have been derived as a statistical means to look for an enrichment of mutations above a background rate for each gene [27, 28], as well as filtering out recurrently mutated genes that are not expressed in a specific organ system [20]. Apart from these purely statistical means, another alternative approach includes focusing on recurrently mutated genes that have been shown to have an effect on cancer causation (such as those recorded in the Cancer Gene Census database http://cancer.sanger.ac.uk/census/). Despite these efforts, there remains controversy as to the functional significance of some frequently mutated genes across diverse cancer types that await validation by functional study, but these are particularly difficult for large frequently mutated genes that are difficult to clone and transfect for in vitro culture assays.

Given the above background, more recent genomic studies generally separate the hypermutated (MSI) cancers and the microsatellite stable (MSS) cancers for independent driver gene analysis because of their marked difference in background mutation rates. Table 21.1 summarizes some of the common putative driver genes

discovered in more recent large-scale genomic studies and their mutational incidence in different molecular subtypes of gastric cancer through combined analysis of these cohorts [19–26]. A few of the newly discovered driver mutations will be discussed in detail in the following section.

1. *Mutation in TP53*—This constitutes the most predominantly mutated driver gene in gastric cancer, with widely different incidence in different molecular subtypes [20]. Mutation is most frequent in intestinal-type gastric cancers, especially the subtype manifesting CIN, with a mutation incidence of 50–70 % [22]. The mutation rate is slightly lower in diffuse-type gastric cancers, and distinctly uncommon in EBV and MSI gastric cancers.
2. *Mutation in ARID1A*—This gene belongs to the SWI-SNF complex of chromatin remodeling genes first discovered to be frequently mutated in gastric cancer by an exome sequencing study from our group [19]. ARID1A is also frequently inactivated by mutation in ovarian clear cell carcinoma and in endometrial cancer [29–31]. Mutations are mostly truncating and accompanied by protein loss. There are also cases of protein loss without underlying mutation, showing there may exist other mechanisms for its inactivation. There is a striking pathway-specific difference in mutational incidence of ARID1A mutation in gastric cancer, with 70–80 % inactivation in gastric cancers with EBV or MSI, and only 11 % in MSS non-EBV cancers [19]. Moreover, the mutation spectrum differs across molecular subtypes. Whereas there are many short mononucleotide repeat tracts in this gene that undergo frameshift mutations in MSI cancers, there are more nonsense mutations or indels involving nonrepeat sequences in MSS cancers [19]. The results suggest a strong selection pressure and hence possible growth advantage for ARID1A mutation taking advantage of the underlying predisposing genomic instability. In general, ARID1A mutation is negative association with TP53 mutation. Functionally, a subsequent study showed that siRNA knockdown of ARID1A in gastric cancer cell lines led to enhanced proliferation [26]. The common mutation of ARID1A in EBV and MSI cancers, both with enrichment in lymphoid infiltrate, may suggest its potential role in immune evasion. Interestingly, apart from ARID1A, mutation of other chromatin remodeling genes are also frequent and enriched in gastric cancer [20], suggesting an important role for chromatin remodeling in gastric carcinogenesis.
3. *Mutation of RHOA in diffuse-type gastric cancer*—Mutation of RHOA was discovered in diffuse-type gastric cancers in three recent genomic studies. Two of the studies, including one by us, first reported RHOA mutation in 14 –25 % of diffuse-type gastric cancers but none in intestinal type [20, 21]. The mutations show clustering at hotspots in the GTP domains and effector binding regions. We have functionally characterized two hotspot mutants, Y42C and L57V, and showed that they result in defective activation of RHOA. Using normal organoid cultures, we found that these mutants being defective in RHO signaling, promote evasion from anoikis [20]. As early phase of diffuse-type gastric cancer development involves breaking off of individual cancer cells through the basement membrane to survive in the lamina propria, defective RHO signaling may facilitate evasion of anoikis which is highly relevant in the carcinogenic process.

Table 21.1 Summary of mutational incidence of key gastric cancer drivers genes and their combined mutational incidence in different molecular subtypes of gastric cancers

Gene symbol	Driver gene nominated	MSS $n=521$ (%)	MSI $n=83$ (%)	EBV $n=38$ (%)	Diffuse $n=215$ (%)	All $n=604$ (%)
TP53	HKWGS/TCGA/ACRG/TIANJIN	**50.5**	33.7	7.9	34.4	48.2
SYNE1	ACRG	14.6	60.2	5.3	13.5	20.9
ARID1A	HKWGS/TCGA/ACRG/TIANJIN	14.8	**80.7**	**57.9**	**17.7**	23.8
CDH1	HKWGS/TCGA/ACRG/TIANJIN	11.7	12.0	0.0	**25.1**	11.8
SPTA1	HKWGS	11.7	32.5	13.2	10.7	14.6
PIK3CA	TCGA/TIANJIN	9.8	**39.8**	**63.2**	11.6	13.9
APC	TCGA/TIANJIN	7.1	31.3	0.0	5.6	10.4
RIMS2	HKWGS	6.5	26.5	5.3	4.7	9.3
GLI3	HKWGS	6.3	28.9	5.3	6.5	9.4
SMAD4	HKWGS/TCGA/TIANJIN	6.3	6.0	**15.8**	4.7	6.3
ERBB4	TIANJIN	6.0	27.7	5.3	5.1	8.9
MUC6	HKWGS/TCGA	5.4	27.7	2.6	4.2	8.4
DCLK1	HKWGS	5.4	19.3	0.0	3.3	7.3
RHOA	HKWGS/TCGA	5.0	3.6	5.3	**10.2**	4.8
THSD7B	HKWGS	4.8	16.9	5.3	5.1	6.5
TSHZ3	TIANJIN	4.4	20.5	0.0	4.2	6.6
MACF1	TCGA	4.4	**45.8**	5.3	2.8	10.1
EPB41L3	ACRG	4.2	21.7	0.0	3.3	6.6
KRAS	TCGA/TIANJIN	4.0	**22.9**	2.6	2.3	6.6
TLR4	TIANJIN	4.0	8.4	2.6	1.9	4.6
LRFN5	ACRG	3.8	9.6	0.0	3.7	4.6
WDFY4	ACRG	3.6	3.6	0.0	3.7	3.6
CTNNA2	HKWGS	3.6	16.9	5.3	4.7	5.5
CTNNB1	TCGA	3.6	10.8	15.8	3.3	4.6
PTPRC	TCGA	3.3	12.0	0.0	2.8	4.5
BNC2	TCGA	3.3	13.3	5.3	1.9	4.6
ERBB2	TCGA	2.9	15.7	2.6	3.3	4.6
ASTN1	ACRG	2.9	13.3	2.6	1.4	4.3
TGFBR2*	ACRG	2.7	18.1	2.6	5.1	4.8
RNF43	HKWGS/TCGA	2.7	**59.0**	0.0	4.2	10.4
NRG1	TIANJIN	2.5	13.3	0.0	1.9	4.0
OPRK1	ACRG	2.5	8.4	2.6	2.8	3.3
RASA1	TCGA	2.3	13.3	2.6	0.5	3.8
THBS1	HKWGS	2.3	13.3	5.3	2.8	3.8
DLGAP2	ACRG	2.1	14.5	0.0	2.8	3.8
ZIC4	HKWGS	1.9	8.4	2.6	2.8	2.8

The list of driver genes is derived from those nominated by four different gastric cancer cohorts [20, 22, 24, 25]. Genes with a combined mutational incidence of <2 % in MSS gastric cancers are excluded. The mutational incidence is derived from a combination of 8 gastric cancer cohorts sequenced by whole-genome or whole-exome sequencing [19–26]. Genes with high mutational incidence in specific molecular subtypes are highlighted. Mutational incidence of TGFBR2 is underestimated in MSI cancers because of the inherent problem in mutation detection algorithm to detect frameshift mutation in a hotspot A10 mononucleotide tract

Functional studies by another group using 3D culture of gastric or other cancer cell lines carrying mutant RHOA showed that siRNA knockdown of RHOA in these cell lines retard growth, whereas siRNA knockdown of RHOA in wild-type cell lines do not show any effect [21]. RHOA mutation was detected in the intramucosal component, as well as deep invasive area [21] and also in early stage cancer [20], suggesting that RHOA mutation occurs early in the carcinogenic event. More interestingly, the recent study by TCGA, apart from finding RHOA mutations, also detected CLDN18-ARHGAP fusions in the genomic stable form of gastric cancers, which is also enriched in diffuse-type gastric cancers [22]. Since ARHGAP functions to convert the active GTP form of RHOA to the inactive GDP form, it is plausible to hypothesize that the fusion with CLDN18, a membrane protein highly expressed in stomach, may result in increased expression of ARHGAP in proximity to the cell membrane, whereby it may lead to increased GTPase activity and inactivation of RHOA, with similar consequence as RHOA mutation. Furthermore, it was found that gastric cancers with RHOA mutation do not overlap with ARHGAP fusion, suggesting they may play a similar role in the carcinogenic process [22].

4. *Mutation of Wnt signaling pathway genes in gastric cancer, including discovery of new driver mutation of RNF43*—The mutational incidence of the APC gene has been widely studied, with discrepancies in mutation incidence and spectrum previously [13, 15]. Recent large-scale genomic studies also revealed varying but overall low incidence of APC gene mutation in gastric cancer. It is mutated in 3–7 % of MSS gastric cancers [20, 22], being more common in intestinal type (10 %) versus 2 % in diffuse type [21]. Mutation of beta-catenin occurs in around 4 % of MSS gastric cancers [20, 22]. Interestingly, another Wnt signaling pathway gene, RNF43, is mutated in 33–60 % of MSI gastric cancers, and 3–5 % in the MSS group [20, 22]. RNF43 is a newly characterized wnt signaling gene, being a ubiquitin ligase induced by wnt, it functions to downregulate wnt signaling by endocytosis of the wnt receptor and target them for degradation [32]. There has been intense interest in this gene with potential therapeutic implication since tumors with mutations in this gene, but wild type for APC mutation, are responsive to wnt inhibitor therapy. This has been observed in pancreatic cancer cell lines carrying RNF43 mutation [33]. The observed high incidence of RNF43 mutation and low incidence of APC mutation, especially in the MSI group of gastric cancers suggest a potential use of Wnt inhibitor as therapy for this group of patients.
5. *Mutation of MUC6*—MUC6 is a gastric-specific mucin expressed in foveolar neck cells, as well as antral glands. It has mucoprotective function. MUC6 is mutated in 6–10 % of MSS gastric cancers and 20 % of MSI cancers. Interestingly, the mutation pattern is enriched for inframe deletions, as well as truncating mutations, consistent with mutational inactivation [20]. Apart from mutation, we also noted downregulated expression of MUC6 in a large proportion of gastric cancers, suggesting an additional mechanism for its inactivation.
6. *Mutation in the KRAS/PIK3CA/PTEN pathway*—Mutation of the PIK3CA gene has been reported to be frequent in MSI gastric cancers (40 %), as well as EBV-

associated gastric cancers (over 60 %), whereas overall in MSS gastric cancers, its mutational incidence is around 10 % [22]. Mutation of KRAS is more commonly observed in MSI gastric cancers (20–30 %) and less so in MSS cancers (4 %). Mutation of PTEN is mainly seen in MSI gastric cancers (13–30 %), but rare in MSS cancers. Mutation of RASA1, a negative regulator of RAS, has been observed in 3 % of MSS gastric cancers [22], suggesting additional mechanisms for activation of this pathway.

7. *Mutation in the CDH1 gene*—Mutation of the CDH1 gene is observed mainly in diffuse-type gastric cancers (25 %) that are microsatellite stable. Interesting, mutation is also frequently observed in MSI gastric cancers (12 %), with indel affecting short repeat sequences in this gene.
8. *TGFB signaling pathway*—Mutation of the SMAD4 gene occurs in approximately 6 % of MSS gastric cancers, whereas frameshift mutations affecting mononucleotide tracts of the TGFBR2 (90 %) and ACVR2A (70 %) are very frequent in MSI cancers. We have also identified infrequent mutation of TGFBR2 and ACVR2A in MSS gastric cancers, as well as frameshift mutations in the ELF3 gene, which is an epithelial-specific transcription factor that can induce TGFBR2 expression. Overall, taking into account these 4 genes, 100 % of MSI and 13 % of MSS gastric cancers show derangement of the TGFB signaling pathway [20]. Notably, germline mutation of SMAD4 is responsible for juvenile polyposis syndrome, with florid hyperplastic polyp development in the stomach, and a propensity for progression to carcinoma, further supporting the important role of SMAD4 in growth suppression in the gastric epithelium.
9. *Other putative driver mutations*—apart from the above genes, numerous other putative driver genes have been identified in various studies, most of which affect around 1–5 % of MSS gastric cancers. These span important cell signaling pathways such as hedgehog (GLI3, ZIC4), ERBB (ERBB2, ERBB3, ERBB4, NRG1), stem cell (DCLK1), cell adhesion (CTNNA2), etc. Pathway analysis revealed that although each of these genes are mutated in a small proportion of cases, together they act in concert to deregulate and target common pathways to achieve the consequence of cancer behavior, underlying the molecular heterogeneity and diversity of gene mutation in gastric cancer.

Mutation Spectrum

Apart from selective driver gene mutations, the large-scale genomic data also provides a unique opportunity to examine the underlying mutational signatures and processes responsible for causing these mutations. Multiple large-scale genomic studies have shown a concordant predominance of two specific mutational signatures in gastric cancer (summarized in Fig. 21.1) [20–22, 24, 25]. One common feature is C>T mutation in the CpG dinucleotide setting. This is the most common type of mutation observed in gastric cancer. This mutation type can arise spontaneously through deamination of the cytosine, leading to its conversion to T. Thus, this

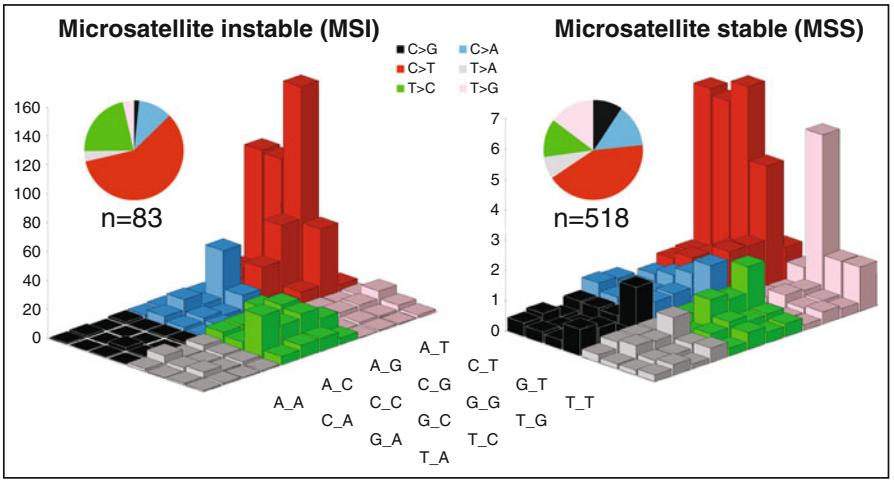


Fig. 21.1 Mutation spectrum in trinucleotide contexts for MSI and MSS gastric cancers. Data is derived from protein altering mutations detected in five large gastric cancer cohorts [20–22, 24, 25]. The mutation spectrum is similar across these different cohorts and hence data are pooled for presentation. Three cases of outliers with specific mutation spectrum dominating and accounting for over 50 % of a specific mutation type of that particular cohort have been removed. The height of each bar represents the mean number of mutations per gastric cancer sample for that particular mutation type

type of mutation can accumulate in cells from birth like a molecular clock, even far before the onset of carcinogenesis. Consistently, we noted a strong correlation of mutational incidence of this type of mutation in relation to age of cancer onset [20]. The other common mutational pattern, T>G mutation, in the TT dinucleotide setting, specifically CTT>CGT, showed an inverse relationship with mutational frequency of C>T mutation, and does not show an association with age, but is significantly associated with antral location in the stomach. Mutations of this type tend to accumulate in the distal part of a gene, and specifically in genes with low transcription rate, thus underlying the mutation process being modulated by transcription-related repair. According to a recent pan-cancer study [34], this mutational signature (known as signature 17), is only seen in cancers arising from stomach, esophagus, liver and B-cell lymphoma, but is rare in all other cancer types.

Structural Variants

Information on structural variants can be derived from whole-genome sequencing studies [20, 22, 24], as well as RNAseq data [22, 35]. Whilst WGS studies, especially with high fold coverage, can give comprehensive information on the frequency and position of these structural variants, including those involving intergenic areas and nontranscribed genes, RNAseq can give more accurate information on the

consequences of the structural variants, specifically on whether they can produce fusion mRNA transcripts that are protein coding. This is because sometimes it is hard to predict how a genomic translocation may affect RNA splicing. Overall, whilst various studies have detected a large number of structural variants in gastric cancer, most of these occur as a singleton event with only rare variants that have been recurrently discovered to generate in-frame protein-coding fusion transcripts. One of the most interesting groups of recurrent fusion genes was discovered by the TCGA study, involving fusion of the 3′ end of two ARHGAP genes (ARHGAP6 and ARHGAP26) to the distal end of the transmembrane protein Claudin 18 [22]. Claudin 18 is a transmembrane protein involved in the formation of tight junctions, highly expressed in gastric epithelial cells, whereas ARHGAP6 and ARHGAP26 are expressed normally at very low levels in the stomach. The fusions invariably affect the 3′-UTR of the CLDN18 gene, thus not immediately obvious to result in in-frame protein-coding fusion products based on the DNA information. However, RNAseq data revealed that the fusions actually activate a cryptic splice site in exon 5 of CLDN18, generating in-frame protein coding fusion transcripts. The breakpoints in ARHGAP6 or ARHGAP26 occur in intronic regions, invariably leading to fusion of the distal GAP domain of ARHGAP to the intracytoplasmic carboxy-terminal of the transmembrane protein CLDN18. As discussed previously, this may lead to increased GTPase activity beneath the cell membrane that could result in increased inactivation of the RHO-GTP form by converting it into GDP form. However, such consequences need to be proven experimentally. The functional significance of this group of CLDN18-ARHGAP fusions is further supported by its prevalence in diffuse type (or genomic stable form) of gastric cancer and its mutual exclusivity with gastric cancers with RHOA mutation. Other recurrent in-frame fusion transcripts involved COL27A1-ZNF618 fusion observed in two gastric cancers [20]. Overall, the lack of many recurrent fusion genes detected may suggest the rarity of such events in gastric cancer, but there are also limitations in the bioinformatics methods used in detecting such structural variants, which may be improved with the availability of better detection algorithms. RNAseq studies can also generate information on alternative splicing, with one study reporting detection of an alternatively spliced form of ZAK isoform that may contribute to the pathogenesis of gastric cancer [35].

Somatic Copy Number Aberration

Studies using DNA array-based technology have generated comprehensive information on chromosomal regions with high level gene amplification, homozygous deletion, as well as copy number gains or losses [20, 22, 36, 37]. The results reveal varying degrees of chromosomal instability, which tend to more severely affect intestinal-type gastric cancers, especially those with TP53 mutation. In these intestinal-type gastric cancers with chromosomal instability, there is extensive aberrations in chromosomal regions, with frequent gains in chromosome 1q, 5p, 7, 8q, 13, and 20; and frequent loss in chromosome 1p, 3p, 4, 5q, 9p, 17p, 18q, 19p, 21, and

22. In the chromosomal stable groups, the most frequent changes involve gain of chromosome 7, 8, 13, and 20. Interestingly, deletion in chromosome 18 is especially common in EBV gastric cancers, whereas MSI gastric cancers show the highest frequency of chromosome 8 gain [20]. The difference in pattern may underlie different requirements for deregulation in these different pathways of carcinogenesis.

High-level gene amplification usually involves putative target oncogenes residing in these amplicons, whereas homozygous deletions target putative tumor suppressors, which can be predicted by the GISTIC2 algorithm. These encompass known oncogenes, such as ERBB2, CCNE1, MET, MYC, CCND1, CDK4, CDK6, FGFR2, EGFR, and KRAS, but also some new putative oncogenes, such as CD44, VEGFA, JAK2, CD274, ZNF217, SUPT3H, PRKC1, and GATA6. Deleted genes include important drivers, such as CDKN2A, PTEN, SMAD4, APC, etc, as well as many new potential tumor suppressor genes, such as ARID1A. Overall, apart from ERBB2, CCNE1, and MYC amplification which are more common (above 10 %), most of the other amplified oncogenes affect only a small subset of gastric cancers (<10 %).

Overall, one main feature of intestinal gastric cancers with TP53 mutations is the prevalence of oncogenic amplifications involving either the cell cycle, receptor tyrosine kinase/RAS pathways, or key transcription factors that help to drive cell proliferation, underlying the importance of chromosomal instability in driving the carcinogenic pathway in this group. There are therapeutic implications that some of these are existing or emerging therapeutic targets, such as ERBB2, FGFR2, MET, and CDK4/6. Interestingly, one study showed that amplification of genes in the RTK/RAS pathway occurs in a mutually exclusive manner and together accounts for 37 % of gastric cancers, and constitute a poor prognostic group [36]. As many of these genes in the RTK/RAS pathway are associated with sensitivity or resistance to therapeutic agents in clinical or pre-clinical development, their assessment have profound implications for development of personalized cancer therapy.

DNA Methylation Profiling

Previous studies on methylation in gastric cancer made used of an earlier version of the Illumina methylation array containing 27,000 probes (Illumina Infinium 27 K methylation array), mostly concentrated in assessing promoter CpG islands, thus covering regions that undergo hypermethylation during the carcinogenic process [38]. Subsequently, a newer version of the methylation array interrogates more than 450,000 CpG sites (Illumina HumanMethylation450 Beadchip), covering also the gene body, intergenic areas, and non-CpG dense regions, thereby enabling the ability to assess areas of the genome that undergo demethylation in the carcinogenic process. Two recent large-scale studies have utilized this latest array to study large cohorts of cancer samples, allowing detailed comparisons across molecular subtypes and providing a unique opportunity to examine both hypermethylation and demethylation events at a genome-wide level [20, 22]. Parallel examination of gene expression in these studies allows for integration to derive genes that are

downregulated by hypermethylation, as well as genes that are upregulated by demethylation. Both studies highlighted the unique pattern of extremely high levels of genome-wide methylation in EBV-associated gastric cancers, with an average of nearly 30,000 probes showing aberrant hypermethylation, constituting 20 to 25 % of all probes in the Illumina array that are unmethylated in normal tissue. Comparisons among ten different tumor types across various organ systems showed that EBV-associated gastric cancers have the highest level of methylation [22]. The second group that displays a high level of aberrant methylation is the MSI group, with an average of around 10,000 probes displaying aberrant methylation, constituting approximately 10 % of all probes unmethylated in normal tissue [20, 22]. A subset of microsatellite stable, non-EBV gastric cancers also displayed similarly high methylation levels, consistent with the existence of a MSS non-EBV CIMP group. In terms of hypomethylation, intestinal-type gastric cancers, especially those with TP53 mutation, showed a high level of demethylation [20]. Whilst hypermethylation tends to occur in promoter CpG islands, demethylation has a predilection for intergenic regions located in the open sea. Demethylation levels were higher in intestinal versus diffuse type. Interestingly, whilst there is a general positive association between the extent of demethylation and chromosome instability, consistent with the previous hypothesis that demethylation may lead to unwinding of the chromatin, making chromosomal breakage at fragile sites more likely, MSI gastric cancers appear to be an exception. Apart from genome-wide hypermethylation, there is also global demethylation in MSI cancers, yet they are chromosome stable [20].

Integration of gene expression and hypermethylation data led to identification of a comprehensive list of genes that are downregulated by promoter methylation [20, 22]. The MLH1 gene is amongst the top candidate genes with the strongest concordant hypermethylation and downregulated expression. Other genes identified include those previously validated to be methylated in cancer with biological implications spanning DNA repair (MGMT), mitotic checkpiont (CHFR), and apoptosis (BNIP3). Moreover, there are many other novel genes with putative functions relevant to carcinogenesis, such as PARP6 (cell cycle) or PDAM (regulate apoptosis), that are silenced by methylation. Because of the inherent high level of methylation, there are also many genes that are silenced predominantly in EBV-associated cancers, many of which may represent unique pathways of viral carcinogenesis. Interestingly, one key gastric driver gene, p16INK4a (CDKN2A), is reported to be silenced by methylation in a majority of EBV gastric cancers [22]. Analysis for methylation and gene expression of this gene is complex, as there are two genes with overlapping transcription in the CDKN2A locus. Thus, examination for exon-specific gene expression encompassing exon 1 (that is unique for p16INK4a) is needed to assess for its transcriptional silencing. Furthermore, there are other mechanisms such as homozygous deletion that can contribute to its downregulated expression. This illustrates the importance of integrative examination of multidimensional genetic and epigenetic mechanisms to understand the extent of perturbation in key driver genes in gastric cancer.

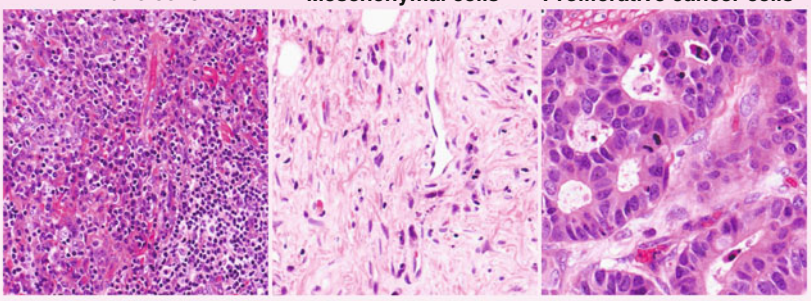
Another aspect of epigenetic dysregulation that is less frequently explored involves demethylation and corresponding upregulated gene expression [20].

Notably, several genes related to intestinal metaplasia, including CDX1, CDH17, TFF3, MUC2, and DFNA5 are upregulated by demethylation in gastric cancer. The ASCL2 gene that plays an important role in conferring a stem cell phenotype is also upregulated by demethylation. Therefore, overall, demethylation may constitute a common alternative mechanism for oncogenic activation.

Gene Expression Profiling

Previous gene expression profiling studies have already identified groups of genes whose coordinated expression reflect intrinsic cellular heterogeneity and signaling pathway alterations in gastric cancer [11, 39] (Fig. 21.2). For example, there are genes predominantly expressed in the extracellular matrix. These include many genes expressed by mesenchymal cells, encoding components of collagen and extracellular matrix (COL1A1, COL1A2, COL3A1, LOX, FN1, SPARC, MYH11, LMOD1), as well as signaling components involved in the TGF-beta (TGFBI) or Wnt (SFRP4) signaling pathways. This group of genes is highly expressed in diffuse-type gastric cancers that have an inherently more aggressive behavior. More recently, renewed interest in this group of genes has been raised because of its significant association with poor prognosis in gastric [37, 40, 41] as well as in other cancer types, including those arising from the colon [42]. Gastric cancers with high expression of this gene cluster have been variably classified and named mesenchymal/stroma [41, 43], EMT [37] or TCGA-C1 [22], which are invariably associated with enrichment for diffuse-type gastric cancers across these different studies. A third cluster of genes is predominantly expressed in tumor infiltrating lymphoid cells, including many T cell markers and cytokine signaling molecules (e.g. CD8A, CD3E, GZMB, GBP1). This group of genes is highly expressed in EBV-associated gastric cancers and to a lesser extent in MSI cases [11], corresponding to cluster C2 in the TCGA study [22]. There is another gene cluster enriched in genes involved in cell cycle and DNA replication (e.g. BUB1, PCNA, CDC2, CDC6), denoted as the proliferation cluster [11], that parallel the rapid proliferation rate of the tumor cells. Gastric cancers enriched in these genes are subsequently termed "Proliferative" [43] or TCGA-C3 [22], and enriched in gastric cancers with CIN and TP53 mutation, which mostly consist of the MSS intestinal type. By contrast, there are genes that are highly expressed in normal gastric mucosa, denoted as the "normal cluster," containing genes encoding normal gastric function, such as PGC, MUC5AC, MUC6, and TFF1. Gastric cancers enriched in this cluster are subsequently termed "metabolic/digestion" [43] or TCGA-C4 [22]. Based on cell line drug screening information, the metabolic subtype is suggested to be more responsive to 5-FU, whereas the mesenchymal subtype is more sensitive to compounds targeting the PI3K-AKT-mTOR pathway [43]. Finally, we have described a gene cluster characterizing intestinal metaplasia, with expression of transcription factors CDX1 and CDX2 that are involved in inducing intestinal differentiation, as well as corresponding intestinal genes such as VIL1, CDH17, HNF4A, etc [11]. Interestingly, this

a



b

Cells	Immune cells	Mesenchymal cells	Proliferative cells	Gastric function	Intestinal function
Representative genes	CD8A, CD3E, GZMB, GBP1, CXCL10	DES, LMOD1, SPARC, COL1A1, LOX, FN1, THBS2, TGFBI, SFRP4	BUB1, PCNA, CDC2, CDC6, CCNE1	PGC, MUC5AC, TFF1, MUC6	CDX1, CDX2, CDH17, VIL1, HNF4A
GC classes based on gene expression in various studies	Cytokine, Immune, TCGA-C2	Mesenchymal, EMT, stromal signature, TCGA-C1	Proliferative, TCGA-C3	Digestion, metabolic, TCGA-C4	Intestinal differentiaton-derived from intestinal metaplasia
Morphological or molecular subtypes	EBV, MSI	MSS-diffuse	Intestinal, MSI		
Prognosis		Poor			
Potential sensitivity to drug	Immune therapy, anti-PDL1	PI3K-AKT-mTOR inhibitors		5-FU	

Fig. 21.2 Gene expression signatures and cell types in gastric cancers. (**a**) Different cell types that exist in gastric cancers which may contribute to different gene expression signatures. (**b**) Gene expression, corresponding signatures, and their relationship with gastric cancer subtypes and therapeutic response [11, 22, 37, 41, 43]

group of genes is expressed in around 50 % of gastric cancers, irrespective of whether there is glandular formation (hence the so-called intestinal type by Lauren's classification) or not. Expression of this group of genes in gastric cancer correlates with the presence of intestinal metaplasia at the tumor edge, suggesting that they represent cancers that have progressed through the intermediate stage of intestinal metaplasia. Finally, a cluster of genes enriched in wnt signaling component (AXIN2, CTNNB1, EPHB2), is consistent with activation of wnt signaling in specific subsets of tumors [11].

Subsequent larger-scale expression profiling or meta-analysis identified genesets or metagenes with processes mostly similar to the above described gene clusters (Fig. 21.2). For example, one study identified metagenes based on a network-based approach, including cell cycle, ECM/stromal, immune response, digestion, mitochondria, proteasome, ribosome, unknown function, and novel [41]. They further described that stromal genes enriched in TGF-beta pathway correlated with the amount of stroma in the tumor, with gastric cancers showing increased stromal gene signature enriched in diffuse type, and associated with poor prognosis. The TCGA

study, based on gene expression data, segregated gastric cancers into four subtypes according to the four clusters of genes most differentially-expressed. Again, these gene clusters reflect key aspects such as cell proliferation, immune response, extracellular matrix, and genes of normal gastric function [22]. The ACRG classified gastric cancers into four subtypes, with the MSS-EMT subtype associated with a poor prognosis [37].

Apart from studying gastric cancer samples, there have also been attempts to derive gene signatures based on the study of gastric cancer cell lines. Through examination of gene expression profiles in 37 gastric cancer cell lines using various types of unsupervised clustering methods, a study has found two intrinsic subtypes of gastric cancers termed G-INT and G-DIF. The gene signature can be used to classify gastric cancer samples, with the G-DIF type having a poorer prognosis. In vitro, gastric cell lines with the G-INT signature are more sensitive to 5-FU and oxaloplatin, whereas the G-DIF type is more sensitive to cisplatin [44].

Lastly, there have been a few larger-scale studies attempting to identify genes or gene signatures predictive of prognosis in gastric cancer using patient survival data [39, 45–47]. Our earlier study, based on univariate cox-regression analysis, has identified some biologically interesting genes whose expression are correlated with good prognosis, including PLA2G2A and CCL18, reflecting intrinsic tumor properties (PLA2G2A) or tumor immune environment (CCL18, expressed by macrophage) [39, 45]. A large-scale study based on paraffin-embedded tissue encompassing 432 gastric cancers (all treated by adjuvant chemoradiotherapy) of different stages with long-term follow-up data has identified 369 probes that are predictive of recurrence [47]. Based on the gradient Lasso algorithm with leave-one-out cross-validation algorithm, the authors built a prognostic algorithm using a 26 gene signature, and found that the signature could segregate patients into low and high risk categories in the early stage (stage IB + II) as well as late stage (stage III + IV) patients. The same signature could also predict prognosis in an independent patient cohort. The authors then refined the gene signature by performing additional studies using a nanostring platform in an independent cohort of 186 stage II gastric cancer patients, and identified an 8-gene signature (LAMP5, CDC25B, CDK1, CLIP4, LTB4R2, MATN3, NOX4, and TFDP1) with a predefined cut-off point that could predict poor outcome. The performance of this gene signature has been validated in an independent set of 216 stage II patients, thus providing a method to identify those 20–25 % of patients that have a high risk of recurrence despite standard adjuvant chemoradiotherapy. Furthermore, application of the signature to another independent set of 300 stage II gastric cancer patients treated by surgery alone was again able to segregate patients into high or low risk for recurrence. It would be most important in future studies to clarify whether the high and low risk patients may independently derive benefit from chemoradiotherapy, which would allow future refinement of treatment protocols based on these risk groups. Interestingly, amongst the eight genes, four of them are associated with poor prognosis and all 4 are expressed in the stroma at higher levels compared to epithelial cancer cells, again suggesting the importance of stromal genes in conferring poor prognosis in gastric cancer.

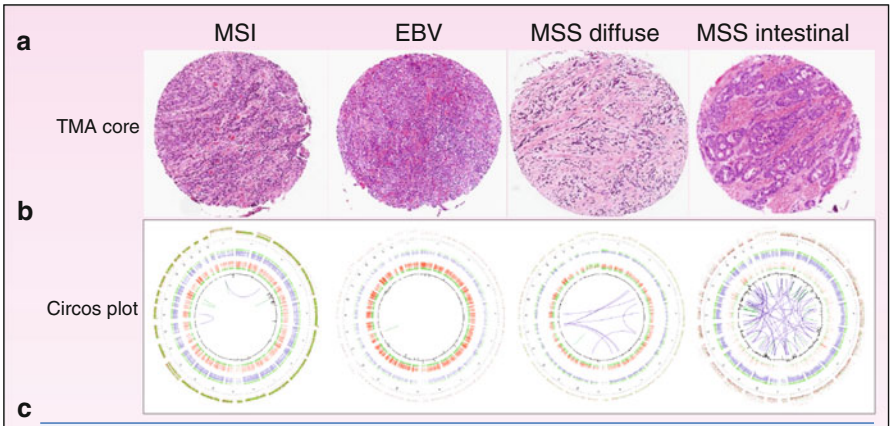
miRNA Expression Profiling

There have been a few large-scale miRNA profiling studies of gastric cancer, some of which have identified microRNA signatures that are predictive of poor prognosis (e.g. low expression of let-7g and miR-433, and high expression of miR-214 [48]; a seven-miRNA signature including miR-10b, miR-21, miR-223, miR-338, let-7a, miR-30a-5p, miR-126 [49]), whereas others showed dysregulated expression of miRNAs with functional relevance to the carcinogenic process, such as downregulation of miR-375 which has pro-apoptotic function [50]. The TCGA study classified miRNA expression into five clusters, with C4 enriched in diffuse or genomically stable type and C2 enriched in the MSI subtype [22], which may again reflect cell type-specific miRNA expression in combination with specific biological functions of each subtypes.

Integrative Genomic Analysis

The availability of comprehensive genomics data reflecting the mutation rate, DNA copy number changes, gene expression, as well as methylation has allowed an unparallel opportunity to understand the kind of perturbations that prevail in different molecular subtypes of gastric cancer as well as combination of driver gene alterations, which would have implication for future approaches to personalized medicine (Fig. 21.3) [20, 22, 37].

One important observation arising out of these studies is the difference in types of genetic and epigenetic perturbations that prevail in specific molecular subtypes of gastric cancer. Specifically, the EBV-associated subtype is characterized by a stable genome, lack of TP53 mutation, prevalent ARID1A mutation, as well as an extensive degree of genome-wide hypermethylation [20]. Frequent PIK3CA mutation, JAK2, as well as PDL1 over-expression also raise potential for targeted drug or immunotherapy for this group of cancers [22]. The MSI subtype is characterized by chromosome stability, hypermethylation and demethylation of the genome, lack of TP53 mutation, and mutation in druggable target genes such as RNF43 and ERBB2 [20]. Intestinal-type gastric cancers are characterized by frequent TP53 mutation, genome-wide hypomethylation, and CIN, which predispose to oncogenic amplification of many oncogenes that are druggable, such as ERBB2, CDK4/6, MET, and KRAS. Finally diffuse-type gastric cancers constitute a distinct group, characterized by a stable genome, unique driver gene alterations that include CDH1, RHOA, CLDN18-ARHGAP fusions, as well as a tendency for promoter CpG hypermethylation [20–22]. This group of tumors is enriched in the mesenchymal phenotype based on gene expression, probably due to the large amount of extracellular matrix present. Whilst each of these morphological or molecularly defined subgroups of GC display well-defined driver gene pathway preferences and genetic or epigenetic perturbations, two recent large-scale studies attempted to derive a molecular classification of gastric cancer based on these genomics features. The TCGA classified gastric cancer into



c

	MSI	EBV	MSS diffuse	MSS intestinal
Location	antrum, body	body, cardia	body, antrum	cardia, antrum, body
Histology	Glands, lymphocytes	Lymphocytes	Diffuse, desmoplastic stroma	Glands
Gene expression	Proliferative, Immune, cytokine	Immune, cytokine, JAK2, PD-L1, PD-L2	Mesenchymal, EMT	Proliferative
DNA copy	CS	CS	CS CLDN18-ARHGAP fusions	CIN Amp - ERBB2, CCNE1, MYC, CDK4/6, MET, KRAS, FGFR2 Del - CDKN2A
Hypermethylation	High level CIMP	Extremely high level CIMP	High level CIMP	
Demethylation	Extensive			Extensive
Key driver	MLH1 methylation with hypermutation	EBV	CDH1, RHO signaling	TP53
TP53 status	WT	WT	MUT/WT	MUT
Other common driver mutations	RNF43, PIK3CA, ERBB2, ARID1A, TGFBR2	ARID1A, PIK3CA	RHOA, CDH1	ARID1A, APC, MUC6

Fig. 21.3 Gastric cancer subtypes and key genomics features. (**a**) Representative histological sections of each subtype. (**b**) Representative *circos plots* of each subtype [20]. *Inner circle* denotes somatic copy number changes and chromosomal translocations; 2nd circle *red dots* denote the beta values of hypermethylated loci; 3rd circle *purple dots* denote the beta values of demethylated loci; *outer circle dots* denotes somatic SNVs. (**c**) Table summarizing key genomic alterations of each subtype, with genes in *red* denoting those with potential therapeutic targets [20, 22]. *CS* chromosome stable, *CIN* chromosomal instability, *Amp* amplification, *Del* deletion, *CIMP* CpG island methylator phenotype, *WT* wild type, *MUT* mutated

four subtypes, the genomic stable, CIN, EBV, and MSI subtypes, with enrichment in putative therapeutic targets in each subgroup [22]. The ACRG classified gastric cancer based on gene expression profiling and gene mutation into the MSI, MSS/TP53+, MSS/TP53-, MSS/EMT subtypes and showed progressively worsening survival according to these 4 groups, and has been able to validate the survival significance in 3 other datasets [37]. Overall, the emerging theme of these classifications still concentrate on a chromosome stable group dominated by diffuse type with lots of extracellular matrix and poor prognosis, the MSI group with good prognosis, the CIN group with intermediate prognosis and marked by oncogenic amplification, an EBV

group with distinct pathogenesis, as well as the potential existence of a TP53 wild-type group that may be associated with a better prognosis. Whether distinction of these subgroups may facilitate future targeted therapy awaits further study.

Linking Genomics Changes to Therapeutic Response

Whilst these genomics studies have revealed a large number of mutations or alterations in gastric cancer, those direct druggable oncogenic targets mainly affect small subsets of patients (mostly less than 10 %). Furthermore, mutation of tumor suppressors predominate, thus it would be important to examine whether there is specific vulnerability associated with specific tumor suppressor deficiency or form of genomic instability. For example, breast cancers with mutation in BRCA genes are sensitive to PARP inhibitor [51, 52], and mutation in PTEN predicts sensitivity to a newly developed class of drugs targeting PLK4 [53]. Recent efforts in large-scale drug sensitivity screening of cancer cell lines have already revealed some interesting drug-gene combinations of sensitivity patterns [54, 55]. A few recent studies have also demonstrated the feasibility of growing different types of cancers using the organoid culture system for large-scale drug screening [56, 57]. These types of studies may eventually lead to a better understanding of the relationship between genomic perturbations and drug response, thereby leading to improved therapeutic efficacy through the development of personalized medicine.

References

1. Leung SY, Yuen ST, Chung LP, Chu KM, Chan AS, Ho JC. hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res. 1999;59:159–64.
2. Leung SY, Yuen ST, Chung LP, Chu KM, Wong MP, Branicki FJ, Ho JC. Microsatellite instability, Epstein-Barr virus, mutation of type II transforming growth factor beta receptor and BAX in gastric carcinomas in Hong Kong Chinese. Br J Cancer. 1999;79:582–8.
3. Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, et al. A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res. 1995;55:5545–7.
4. Yuen ST, Chung LP, Leung SY, Luk IS, Chan SY, Ho J. In situ detection of Epstein-Barr virus in gastric and colorectal adenocarcinomas. Am J Surg Pathol. 1994;18:1158–63.
5. Shibata D, Weiss LM. Epstein-Barr virus-associated gastric adenocarcinoma. Am J Pathol. 1992;140:769–74.
6. Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, Tanaka S, Sato E, Osato T. Gastric carcinoma: monoclonal epithelial malignant cells expressing Epstein-Barr virus latent infection protein. Proc Natl Acad Sci U S A. 1994;91:9131–5.
7. dos Santos NR, Seruca R, Constancia M, Seixas M, Sobrinho-Simoes M. Microsatellite instability at multiple loci in gastric carcinoma: clinicopathologic implications and prognosis. Gastroenterology. 1996;110:38–44.

8. Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M. CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. Clin Cancer Res. 2006;12:2995–3002.
9. Kusano M, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, Hosokawa M, Shinomura Y, Imai K, Tokino T. Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. Cancer. 2006;106:1467–79.
10. Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa JP. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res. 1999;59:5438–42.
11. Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, Chan AS, Law S, Troyanskaya OG, Wong J, So S, Botstein D, Brown PO. Variation in gene expression patterns in human gastric cancers. Mol Biol Cell. 2003;14:3208–15.
12. Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR, Hofler H. E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res. 1994;54:3845–52.
13. Nakatsuru S, Yanagisawa A, Ichii S, Tahara E, Kato Y, Nakamura Y, Horii A. Somatic mutation of the APC gene in gastric cancer: frequent mutations in very well differentiated adenocarcinoma and signet-ring cell carcinoma. Hum Mol Genet. 1992;1:559–63.
14. Park WS, Oh RR, Park JY, Lee SH, Shin MS, Kim YS, Kim SY, Lee HK, Kim PJ, Oh ST, Yoo NJ, Lee JY. Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. Cancer Res. 1999;59:4257–60.
15. Powell SM, Cummings OW, Mullen JA, Asghar A, Fuga G, Piva P, Minacci C, Megha T, Tosi P, Jackson CE. Characterization of the APC gene in sporadic gastric adenocarcinomas. Oncogene. 1996;12:1953–9.
16. Cimerman M, Repse S, Jelenc F, Omejc M, Bitenc M, Lamovec J. Comparison of Lauren's, Ming's and WHO histological classifications of gastric cancer as a prognostic factor for operated patients. Int Surg. 1994;79:27–32.
17. Camargo MC, Kim WH, Chiaravalli AM, Kim KM, Corvalan AH, Matsuo K, Yu J, Sung JJ, Herrera-Goepfert R, Meneses-Gonzalez F, Kijima Y, Natsugoe S, Liao LM, Lissowska J, Kim S, Hu N, Gonzalez CA, Yatabe Y, Koriyama C, Hewitt SM, Akiba S, Gulley ML, Taylor PR, Rabkin CS. Improved survival of gastric cancer with tumour Epstein-Barr virus positivity: an international pooled analysis. Gut. 2014;63:236–43.
18. Choi YY, Bae JM, An JY, Kwon IG, Cho I, Shin HB, Eiji T, Aburahmah M, Kim HI, Cheong JH, Hyung WJ, Noh SH. Is microsatellite instability a prognostic marker in gastric cancer? A systematic review with meta-analysis. J Surg Oncol. 2014;110:129–35.
19. Wang K, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J, Leung SY. Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nat Genet. 2011;43:1219–23.
20. Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, Chan KH, Chan AS, Tsui WY, Ho SL, Chan AK, Man JL, Foglizzo V, Ng MK, Chan AS, Ching YP, Cheng GH, Xie T, Fernandez J, Li VS, Clevers H, Rejto PA, Mao M, Leung SY. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014;46:573–82.
21. Kakiuchi M, Nishizawa T, Ueda H, Gotoh K, Tanaka A, Hayashi A, Yamamoto S, Tatsuno K, Katoh H, Watanabe Y, Ichimura T, Ushiku T, Funahashi S, Tateishi K, Wada I, Shimizu N, Nomura S, Koike K, Seto Y, Fukayama M, Aburatani H, Ishikawa S. Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet. 2014;46:583–7.
22. Cancer Genome Atlas Research N. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.
23. Lee YS, Cho YS, Lee GK, Lee S, Kim YW, Jho S, Kim HM, Hong SH, Hwang JA, Kim SY, Hong D, Choi IJ, Kim BC, Kim BC, Kim CH, Choi H, Kim Y, Kim KW, Kong G, Kim HL, Bhak J, Lee SH, Lee JS. Genomic profile analysis of diffuse-type gastric cancers. Genome Biol. 2014;15:R55.

24. Wong SS, Kim KM, Ting JC, Yu K, Fu J, Liu S, Cristescu R, Nebozhyn M, Gong L, Yue YG, Wang J, Ronghua C, Loboda A, Hardwick J, Liu X, Dai H, Jin JG, Ye XS, Kang SY, Do IG, Park JO, Sohn TS, Reinhard C, Lee J, Kim S, Aggarwal A. Genomic landscape and genetic heterogeneity in gastric adenocarcinoma revealed by whole-genome sequencing. Nat Commun. 2014;5:5477.
25. Chen K, Yang D, Li X, Sun B, Song F, Cao W, Brat DJ, Gao Z, Li H, Liang H, Zhao Y, Zheng H, Li M, Buckner J, Patterson SD, Ye X, Reinhard C, Bhathena A, Joshi D, Mischel PS, Croce CM, Wang YM, Raghavakaimal S, Li H, Lu X, Pan Y, Chang H, Ba S, Luo L, Cavenee WK, Zhang W, Hao X. Mutational landscape of gastric adenocarcinoma in Chinese: implications for prognosis and therapy. Proc Natl Acad Sci U S A. 2015;112:1107–12.
26. Zang ZJ, Cutcutache I, Poon SL, Zhang SL, McPherson JR, Tao J, Rajasegaran V, Heng HL, Deng N, Gan A, Lim KH, Ong CK, Huang D, Chin SY, Tan IB, Ng CC, Yu W, Wu Y, Lee M, Wu J, Poh D, Wan WK, Rha SY, So J, Salto-Tellez M, Yeoh KG, Wong WK, Zhu YJ, Futreal PA, Pang B, Ruan Y, Hillmer AM, Bertrand D, Nagarajan N, Rozen S, Teh BT, Tan P. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet. 2012;44:570–4.
27. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, Kiezun A, Hammerman PS, McKenna A, Drier Y, Zou L, Ramos AH, Pugh TJ, Stransky N, Helman E, Kim J, Sougnez C, Ambrogio L, Nickerson E, Shefler E, Cortes ML, Auclair D, Saksena G, Voet D, Noble M, DiCara D, Lin P, Lichtenstein L, Heiman DI, Fennell T, Imielinski M, Hernandez B, Hodis E, Baca S, Dulak AM, Lohr J, Landau DA, Wu CJ, Melendez-Zajgla J, Hidalgo-Miranda A, Koren A, McCarroll SA, Mora J, Lee RS, Crompton B, Onofrio R, Parkin M, Winckler W, Ardlie K, Gabriel SB, Roberts CW, Biegel JA, Stegmaier K, Bass AJ, Garraway LA, Meyerson M, Golub TR, Gordenin DA, Sunyaev S, Lander ES, Getz G. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499:214–8.
28. Youn A, Simon R. Identifying cancer driver genes in tumor genome sequencing studies. Bioinformatics. 2011;27:175–81.
29. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih Ie M, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG. ARID1A mutations in endometriosis-associated ovarian carcinomas. N Engl J Med. 2010;363:1532–43.
30. Jones S, Wang TL, Shih Ie M, Mao TL, Nakayama K, Roden R, Glas R, Slamon D, Diaz Jr LA, Vogelstein B, Kinzler KW, Velculescu VE, Papadopoulos N. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science. 2010;330:228–31.
31. Guan B, Mao TL, Panuganti PK, Kuhn E, Kurman RJ, Maeda D, Chen E, Jeng YM, Wang TL, Shih Ie M. Mutation and loss of expression of ARID1A in uterine low-grade endometrioid carcinoma. Am J Surg Pathol. 2011;35:625–32.
32. Koo B-K, Spit M, Jordens I, Low TY, Stange DE, van de Wetering M, van Es JH, Mohammed S, Heck AJR, Maurice MM, Clevers H. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. Nature. 2012;488:665–9.
33. Jiang X, Hao H-X, Growney JD, Woolfenden S, Bottiglio C, Ng N, Lu B, Hsieh MH, Bagdasarian L, Meyer R, Smith TR, Avello M, Charlat O, Xie Y, Porter JA, Pan S, Liu J, McLaughlin ME, Cong F. Inactivating mutations of RNF43 confer Wnt dependency in pancreatic ductal adenocarcinoma. Proc Natl Acad Sci. 2013;110:12649–54.
34. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio, SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinsk M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van

Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR, Zucman-Rossi J, Andrew Futreal P, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature. 2013;500:415–21.

35. Liu J, McCleland M, Stawiski EW, Gnad F, Mayba O, Haverty PM, Durinck S, Chen YJ, Klijn C, Jhunjhunwala S, Lawrence M, Liu H, Wan Y, Chopra V, Yaylaoglu MB, Yuan W, Ha C, Gilbert HN, Reeder J, Pau G, Stinson J, Stern HM, Manning G, Wu TD, Neve RM, de Sauvage FJ, Modrusan Z, Seshagiri S, Firestein R, Zhang Z. Integrated exome and transcriptome sequencing reveals ZAK isoform usage in gastric cancer. Nat Commun. 2014;5:3830.
36. Deng N, Goh LK, Wang H, Das K, Tao J, Tan IB, Zhang S, Lee M, Wu J, Lim KH, Lei Z, Goh G, Lim QY, Tan AL, Sin Poh DY, Riahi S, Bell S, Shi MM, Linnartz R, Zhu F, Yeoh KG, Toh HC, Yong WP, Cheong HC, Rha SY, Boussioutas A, Grabsch H, Rozen S, Tan P. A comprehensive survey of genomic alterations in gastric cancer reveals systematic patterns of molecular exclusivity and co-occurrence among distinct therapeutic targets. Gut. 2012;61:673–84.
37. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M, Hongyue D, Reinhard C, Loboda A, Kim S, Aggarwal A. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21:449–56.
38. Zouridis H, Deng N, Ivanova T, Zhu Y, Wong B, Huang D, Wu YH, Wu Y, Tan IB, Liem N, Gopalakrishnan V, Luo Q, Wu J, Lee M, Yong WP, Goh LK, Teh BT, Rozen S, Tan P. Methylation subtypes and large-scale epigenetic alterations in gastric cancer. Sci Transl Med. 2012;4:156ra140.
39. Leung SY, Chen X, Chu KM, Yuen ST, Mathy J, Ji J, Chan AS, Li R, Law S, Troyanskaya OG, Tu IP, Wong J, So S, Botstein D, Brown PO. Phospholipase A2 group IIA expression in gastric adenocarcinoma is associated with prolonged survival and less frequent metastasis. Proc Natl Acad Sci U S A. 2002;99:16203–8.
40. Busuttil RA, George J, Tothill RW, Ioculano K, Kowalczyk A, Mitchell C, Lade S, Tan P, Haviv I, Boussioutas A. A signature predicting poor prognosis in gastric and ovarian cancer represents a coordinated macrophage and stromal response. Clin Cancer Res. 2014;20:2761–72.
41. Wu Y, Grabsch H, Ivanova T, Tan IB, Murray J, Ooi CH, Wright AI, West NP, Hutchins GG, Wu J, Lee M, Lee J, Koo JH, Yeoh KG, van Grieken N, Ylstra B, Rha SY, Ajani JA, Cheong JH, Noh SH, Lim KH, Boussioutas A, Lee JS, Tan P. Comprehensive genomic meta-analysis identifies intra-tumoural stroma as a predictor of survival in patients with gastric cancer. Gut. 2013;62:1100–11.
42. Calon A, Lonardo E, Berenguer-Llergo A, Espinet E, Hernando-Momblona X, Iglesias M, Sevillano M, Palomo-Ponce S, Tauriello DV, Byrom D, Cortina C, Morral C, Barcelo C, Tosi S, Riera A, Attolini CS, Rossell D, Sancho E, Batlle E. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. Nat Genet. 2015;47:320–9.
43. Lei Z, Tan IB, Das K, Deng N, Zouridis H, Pattison S, Chua C, Feng Z, Guan YK, Ooi CH, Ivanova T, Zhang S, Lee M, Wu J, Ngo A, Manesh S, Tan E, Teh BT, So JB, Goh LK, Boussioutas A, Lim TK, Flotow H, Tan P, Rozen SG. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. Gastroenterology. 2013;145:554–65.
44. Tan IB, Ivanova T, Lim KH, Ong CW, Deng N, Lee J, Tan SH, Wu J, Lee MH, Ooi CH, Rha SY, Wong WK, Boussioutas A, Yeoh KG, So J, Yong WP, Tsuburaya A, Grabsch H, Toh HC, Rozen S, Cheong JH, Noh SH, Wan WK, Ajani JA, Lee JS, Tellez MS, Tan P. Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy. Gastroenterology. 2011;141:476–85. 485e471–411.
45. Leung SY, Yuen ST, Chu KM, Mathy JA, Li R, Chan AS, Law S, Wong J, Chen X, So S. Expression profiling identifies chemokine (C-C motif) ligand 18 as an independent prognostic indicator in gastric cancer. Gastroenterology. 2004;127:457–69.
46. Cho JY, Lim JY, Cheong JH, Park YY, Yoon SL, Kim SM, Kim SB, Kim H, Hong SW, Park YN, Noh SH, Park ES, Chu IS, Hong WK, Ajani JA, Lee JS. Gene expression signature-based prognostic risk score in gastric cancer. Clin Cancer Res. 2011;17:1850–7.

47. Lee J, Sohn I, Do IG, Kim KM, Park SH, Park JO, Park YS, Lim HY, Sohn TS, Bae JM, Choi MG, Lim Do H, Min BH, Lee JH, Rhee PL, Kim JJ, Choi DI, Tan IB, Das K, Tan P, Jung SH, Kang WK, Kim S. Nanostring-based multigene assay to predict recurrence for gastric cancer patients after surgery. PloS One. 2014;9:e90133.
48. Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, Yoshida K, Sasaki H, Nomura S, Seto Y, Kaminishi M, Calin GA, Croce CM. Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 2010;11:136–46.
49. Li X, Zhang Y, Ding J, Wu K, Fan D. Survival prediction of gastric cancer by a seven-microRNA signature. Gut. 2010;59:579–85.
50. Tsukamoto Y, Nakada C, Noguchi T, Tanigawa M, Nguyen LT, Uchida T, Hijiya N, Matsuura K, Fujioka T, Seto M, Moriyama M. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. Cancer Res. 2010;70:2339–49.
51. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, Helleday T. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005;434:913–7.
52. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434:917–21.
53. Mason JM, Lin DC, Wei X, Che Y, Yao Y, Kiarash R, Cescon DW, Fletcher GC, Awrey DE, Bray MR, Pan G, Mak TW. Functional characterization of CFI-400945, a Polo-like kinase 4 inhibitor, as a potential anticancer agent. Cancer Cell. 2014;26:163–76.
54. Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW, Greninger P, Thompson IR, Luo X, Soares J, Liu Q, Iorio F, Surdez D, Chen L, Milano RJ, Bignell GR, Tam AT, Davies H, Stevenson JA, Barthorpe S, Lutz SR, Kogera F, Lawrence K, McLaren-Douglas A, Mitropoulos X, Mironenko T, Thi H, Richardson L, Zhou W, Jewitt F, Zhang T, O'Brien P, Boisvert JL, Price S, Hur W, Yang W, Deng X, Butler A, Choi HG, Chang JW, Baselga J, Stamenkovic I, Engelman JA, Sharma SV, Delattre O, Saez-Rodriguez J, Gray NS, Settleman J, Futreal PA, Haber DA, Stratton MR, Ramaswamy S, McDermott U, Benes CH. Systematic identification of genomic markers of drug sensitivity in cancer cells. Nature. 2012;483:570–5.
55. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehar J, Kryukov GV, Sonkin D, Reddy A, Liu M, Murray L, Berger MF, Monahan JE, Morais P, Meltzer J, Korejwa A, Jane-Valbuena J, Mapa FA, Thibault J, Bric-Furlong E, Raman P, Shipway A, Engels IH, Cheng J, Yu GK, Yu J, Aspesi Jr P, de Silva M, Jagtap K, Jones MD, Wang L, Hatton C, Palescandolo E, Gupta S, Mahan S, Sougnez C, Onofrio RC, Liefeld T, MacConaill L, Winckler W, Reich M, Li N, Mesirov JP, Gabriel SB, Getz G, Ardlie K, Chan V, Myer VE, Weber BL, Porter J, Warmuth M, Finan P, Harris JL, Meyerson M, Golub TR, Morrissey MP, Sellers WR, Schlegel R, Garraway LA. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 2012;483:603–7.
56. Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, Dowling C, Wanjala JN, Undvall EA, Arora VK, Wongvipat J, Kossai M, Ramazanoglu S, Barboza LP, Di W, Cao Z, Zhang QF, Sirota I, Ran L, MacDonald TY, Beltran H, Mosquera JM, Touijer KA, Scardino PT, Laudone VP, Curtis KR, Rathkopf DE, Morris MJ, Danila DC, Slovin SF, Solomon SB, Eastham JA, Chi P, Carver B, Rubin MA, Scher HI, Clevers H, Sawyers CL, Chen Y. Organoid cultures derived from patients with advanced prostate cancer. Cell. 2014;159:176–87.
57. van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, McLaren-Douglas A, Blokker J, Jaksani S, Bartfeld S, Volckman R, van Sluis P, Li VS, Seepo S, Sekhar Pedamallu C, Cibulskis K, Carter SL, McKenna A, Lawrence MS, Lichtenstein L, Stewart C, Koster J, Versteeg R, van Oudenaarden A, Saez-Rodriguez J, Vries RG, Getz G, Wessels L, Stratton MR, McDermott U, Meyerson M, Garnett MJ, Clevers H. Prospective derivation of a living organoid biobank of colorectal cancer patients. Cell. 2015;161:933–45.

Chapter 22
Recapitulating Human Gastric Cancer Pathogenesis: Experimental Models of Gastric Cancer

Lin Ding, Mohamad El Zaatari, and Juanita L. Merchant

Overview

Gastric cancer has been traditionally defined by the Correa paradigm as a progression of sequential pathological events that begins with chronic inflammation [1]. Infection with *Helicobacter pylori* (*H. pylori*) is the typical explanation for why the stomach becomes chronically inflamed. Acute gastric inflammation then leads to chronic gastritis, atrophy particularly of acid-secreting parietal cells, metaplasia due to mucous neck cell expansion from trans-differentiation of zymogenic cells to dysplasia and eventually carcinoma [2]. The chapter contains an overview of gastric anatomy and physiology to set the stage for signaling pathways that play a role in gastric tumorigenesis. Finally, the major known mouse models of gastric transformation are critiqued in terms of the rationale behind their generation and contribution to our understanding of human cancer subtypes.

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Comparative Gastric Anatomy and Physiology

Gastric Anatomy

The stomach is surrounded by the greater and lesser omenta, which both provide conduits for draining lymph nodes and lymphatic vessels, blood vessels, and nerves. The lesser omentum supports the lesser curvature of the stomach and anchors it to the liver. The greater omentum emerges to overlie the small intestinal tube and supports the greater curvature of the stomach. Cancer cells can therefore drain into the supporting lymph nodes or can be transported through the gastric and/or gastro-omental veins, which all lead to the hepatic portal vein into the liver. There are multiple clusters of lymph nodes draining the stomach, which are supported by the omenta. For example, pathogenic antigens from *Helicobacter pylori* or Epstein Barr Virus (EBV) in theory drain from the mucosa into lymph nodes via the afferent lymphatics or postcapillary high-endothelial venules to activate B cell germination, plasma cell generation, and antibody production. Concurrently, the gastric mucosa and submucosa are invaded by a large influx of immune cells including monocytes, macrophages, dendritic cells, neutrophils, B and T effector cells, T-regulatory cells, and mast cells. However, the relationship between the initiation of gastric inflammation in the mucosa and its dependence on antigen presentation in the lymph nodes is poorly understood and might contribute to the difficulty in generating a cancer-preventing vaccine.

Histology

It is important to outline the cellular layers of the gastric tube in order to understand the pre-malignant developments that were outlined by Correa [1]. The human stomach is divided into four parts which display different histological characteristics: (1) cardia, (2) fundus, (3) corpus or body, and (4) antrum/pylorus. Mice lack a cardia but contain two different glandular domains (the body and the antrum). The gastric tube is composed of mucosa (inner epithelial lining facing the lumen), a submucosa formed of dense connective tissue, three layers of muscle (inner oblique, middle circular, and outer longitudinal), and serosa. The muscularis mucosa is a thin layer of smooth muscle that separates the mucosa from submucosal layers (Fig. 22.1). The epithelial mucosa is organized into glands, which vary in their cellular composition between different parts of the stomach.

Gastric Cardia

The gastric cardia lies adjacent to the gastroesophageal junction and consists of tortuous glands populated by mucous-secreting pit cells and scattered oxyntic and chief cells in a 1:1 pit to gland ratio. The main function of the cardia is to neutralize the acidic content of the stomach adjacent to the gastroesophageal junction.

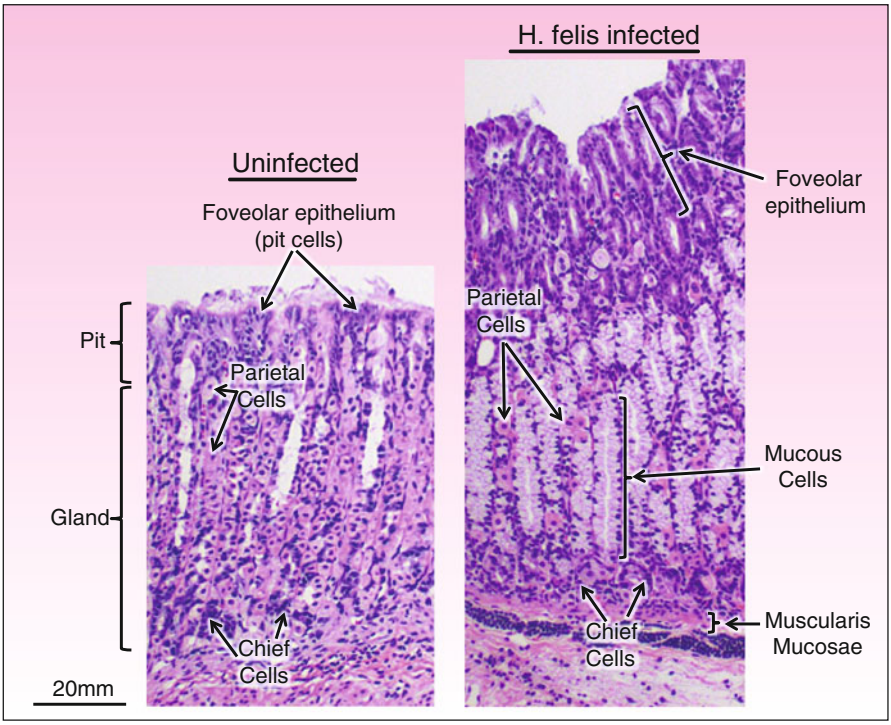

Fig. 22.1 Histological structures of the gastric body of the mouse in normal and inflamed mucosal epithelia. Cell types resemble those of the human stomach. *Left panel*, normal uninflamed gastric mucosa. *Right panel*, chronically inflamed mucosa after 6-month *Helicobacter felis* (H. felis) infection. Annotated are the gastric pits and glands, and constituent cell types, including: (1) pit cells (foveolar cells), (2) parietal cells (large eosinophilic cells which are "fried egg"-shaped), (3) chief/zymogenic cells (basophilic cells at base of the gland), (4) mucous neck cells, and (5) smooth muscle cells of the muscularis mucosa

This function depends on mucin- and bicarbonate-rich secretions by the mucous pit and neck cells. The gastric cardia is associated with gastroesophageal acid reflux disease (GERD*)* and gastric cardia cancer [3]. Gastric cardia cancer is currently on the rise in the US for unknown reasons, but epidemiologically this cancer correlates with inflammation-driven gastric atrophy and acid-bile reflux [4, 5].

Gastric Fundus and Corpus

These two anatomical regions display a more heterogeneous composition than the cardia. The epithelial mucosa consists of a mixture of glands that exhibit a shorter pit cell region with a pit to gland ratio of 1:4 or 1:5, respectively. The fundus and corpus contain several major cell types: (1) acid-secreting parietal cells spanning the entire central gland region, (2) pit cells (mucus-secreting), (3) neck cells

(mucus-secreting), (4) zymogenic or chief cells (pepsinogen and lipase-secreting), and (5) endocrine cells that secrete various bioamines or peptide hormones (Fig. 22.1). These cells play several physiological roles.

The parietal cells exchange hydrogen for potassium ions using ATP (H^+,K^+-ATPase) from abundant mitochondria that fill their cytoplasm. Parietal cells contain a tubulovesicular membrane network available to increase the plasma membrane surface area at the apical surface upon secretagogue stimulation. During secretion, the tubulovesicular membrane organizes into apically directed canaliculi simultaneously with insertion of the H^+,K^+-ATPase enzyme. The rich membranous content and mitochondrial overabundance imparts to parietal cells their distinctive eosinophilic hue on H&E stains and coupled with their large size (~10 μm) gives these cells a "fried egg" appearance (Fig. 22.1). Pathologically, these cells are very important in the innate mucosal protection against pathogens due to their acid-secreting capabilities. It is therefore not surprising that their loss (atrophy) signals one of the earliest events during *H. pylori*-induced chronic gastritis. Whether triggered by a pathogen or a chronic immunological defect, parietal cell atrophy is a common occurrence that precedes malignant development (Figs. 22.1 and 22.2).

The fundic surface pit and neck cells are mucus-secreting, and like the cardia, these cells secrete large amounts of mucins and bicarbonate-rich secretions to neutralize the effects of stomach acid. These cells expand in response to chronic inflammation at the expense of parietal cell atrophy (Fig. 22.1). In mice, they arise from cryptic progenitor stem cells residing in the chief cell layer at the base of the fundic gland [6]. Transdifferentiation of these cells into hybrid chief/mucous cells signals the development of gastric metaplasia, which is believed to precede the development of the differentiated gastric cancer subtype. In mice, the metaplasia

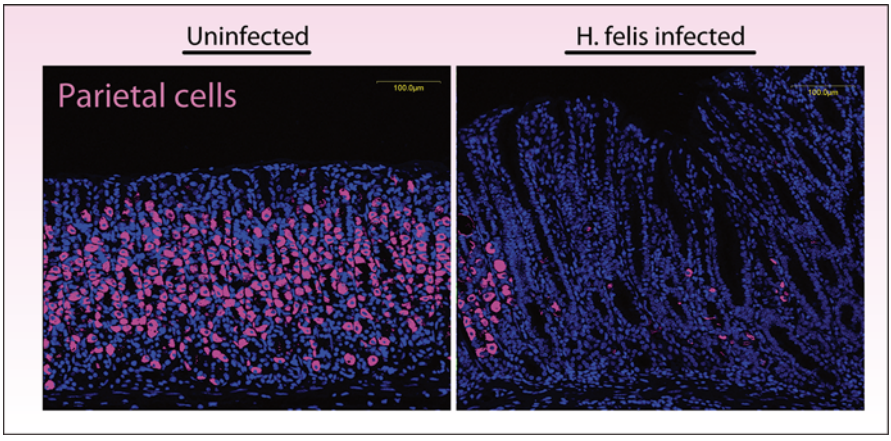

Fig. 22.2 Loss of parietal cells (*pink*) following chronic (>6 months) *H. felis* infection. Immunofluorescent photomicrographs of gastric corpus mucosa showing parietal cells. Parietal cells stained in pink in normal uninfected gastric mucosa (*left panel*) and chronically (>6 months) infected mucosa with *H. felis* (*right panel*)

expresses trefoil factor 2 (TFF2), also known as spasmolytic polypeptide. Therefore the mouse form of gastric metaplasia is called SPEM for SP-Expressing Metaplasia [7]. SPEM also develops in the human stomach, but more typically is described as intestinal metaplasia in which the gastric metaplasia resembles goblet cells of the small intestine (complete intestinal metaplasia) or colon (incomplete metaplasia) [8, 9].

The chief or zymogenic cells located at the base of fundic glands secrete lipase- and pepsinogen. Acid produced by parietal cells stimulates the activation of zymogenic enzymes produced by chief cells, for example by hydrolysis of pepsinogen to pepsin. Due to their protein-secreting properties, chief cells contain a large amount of rough endoplasmic reticula, giving these cells a strong basophilic appearance with H&E staining (Fig. 22.1). Electron microscopy of these cells shows an abundance of secretory vesicles at the apical surface indicating luminal secretion. In addition, a subset of zymogenic cells harbors a cryptic progenitor or "stem cell" that transdifferentiates into SPEM during chronic gastric inflammation [6]. Indeed another report showed that a subset of zymogenic cells express the stem cell marker Troy and give rise to entire gastric units thereby confirming their progenitor capability [10].

The endocrine cells of the fundus/corpus consist of the Delta (D) and Enterochromaffin-like (ECL) cells, which express muscarinic M_3 receptors. Acetylcholine directly stimulates the D, ECL, and parietal cells to secrete somatostatin, histamine, and acid respectively. Somatostatin from D cells also indirectly regulates parietal cell acid secretion through paracrine stimulation of ECL cells to produce histamine. Thus ECL cells express somatostatin receptors while parietal cells express histamine receptors [11]. Endocrine cells play an important role in gastric pathology by regulating the output of acid secretion, and therefore affect development of hypochlorhydria produced during chronic gastritis.

Gastric Antrum

The gastric antrum displays a more homogenous composition of mucous glands with a 2:1 pit to gland ratio. Antrum function is epitomized by its prominent endocrine role due to the presence of the gastrin-producing G and somatostatin-producing D cells. Unlike the D cells of the fundus (closed-type), the D cells of the antrum are open to the gastric lumen (open-type). The antral D cells therefore sense the acidic-luminal content which stimulates the paracrine release of somatostatin. Activated somatostatin receptors on the antral G cell inhibit gastrin gene expression and secretion [12, 13]. G cells are only present in the antrum where their apical surface faces the lumen to sense digested amino acids in the gastric chyme, probably through primary cilia [14, 15]. G cells respond to several stimuli including: (1) luminal content, (2) parasympathetic stimulation by gastrin-releasing peptide (GRP) secreted from postganglionic fibers of the vagus nerve and, (3) somatostatin inhibition. G cells secrete gastrin basolaterally into the circulation,

which then targets cells in the fundus, e.g., stem cells, parietal cells, and D cells in the antrum to complete the negative feedback loop. The importance of gastrin in gastric cancer has been exploited in mouse models of hypergastrinemia, which develop pre-malignant lesions due to chronic stimulation by high gastrin levels which exerts a proliferative effect on mucous pit, parietal and ECL cells in the fundus, but not the antrum [16].

Histologic and Molecular Classification of Gastric Cancer

EBV-associated cancers exhibit higher CpG island methylation associated with mutations in the alpha subunit of the PI3K enzyme (*PI3KCA*). Growth factor pathways, e.g., EGFR and mitotic pathways were commonly perturbed in the MSI cancers; whereas p53 was the most prominent gene abnormality in CIN tumors. Ninety percent of gastric cancers are adenocarcinomas (American Cancer Society: Cancer Facts and Figures 2015; http://www.cancer.org/acs/groups/content/@editorial/documents/document/acspc-044552.pdf). However, other cell types can develop into cancer including a B cell lymphoma called mucosa-associated lymphoid tissue (MALT), or neuroendocrine-related tumors arising from ECL cells due to hypergastrinemia (type 1 and 2 gastric carcinoids) [17, 18]. Gastric adenocarcinomas are histologically classified into two types according to the Lauren classification: differentiated or diffuse [19]. Cancers classified as differentiated or intestinal-type arise in the setting of chronic inflammation as described by Correa [1]. On the other hand, the diffuse type exhibits *dis-cohesive* expansion of mucus-secreting cells and is poorly differentiated (lack organized glandular features). In some instances of diffuse gastric cancer, mucus is retained within the tumor cell and displaces the nucleus to the periphery, producing what is known as signet-ring cell carcinoma.

Although the mechanisms leading to the different types of adenocarcinoma remain unclear, recent studies have reclassified gastric cancers according to their molecular signatures [20, 21]. For example, The Cancer Genome Atlas (TCGA) classified gastric adenocarcinomas as: (1) EBV-associated (EBV); (2) microsatellite instability (MSI); (3) genomically stable (GS); (4) chromosomal instability (CIN) [20]. Moreover, these analyses provided valuable insight into some of the molecular mechanisms that underlie different histological subtypes. For example, the diffuse gastric cancer subtype was enriched in the GS group, which contains mutations in the RHOA, CDH1 genes, or a CLDN18-ARHGAP26 translocation, all loci associated with the cell cytoskeleton [20]. By contrast differentiated gastric cancer subtypes are enriched in the EBV, MSI, and CIN subgroups [20]. Recently, the Asian Cancer Research Group reported gastric cancer classification based upon p53 activity (MDM2 and $p21^{Waf1}$ expression) [22]. Although their whole genome sequencing of 251 gastric cancers validated the TCGA classifications, their collection of added clinical data permitted further correlation of genotypes with p53 status. Interestingly, subjects with microsatellite stable (MSS) versus microsatellite instability (MSI)

tumors showed worse survival. Within the MSS group, those cancers that loss p53 activity or exhibited epithelial–mesenchymal transition (EMT) exhibited the poorest survival. Thus, determining the underlying mechanisms for each molecular subtype is now important to further understand the etiology of these cancers.

Contribution from Invertebrate Biology

Drosophila midgut flexibly enables the genetic modeling of gastric stem cells. The *Drosophila* gut contains a region of low pH (<3) that resembles the mammalian stomach. This area was initially identified by its characteristic ability to accumulate copper [23, 24], and hence the designation "copper cells region" (CCR). The CCR contains three different cell types: (1) copper cells, (2) interstitial cells, and (3) enteroendocrine cells. Copper cells resemble parietal cells in their structure and function: they contain an invaginated apical membrane with microvilli and H^+-ATPase pumps (that recapitulates parietal cell canaliculi), with an abundance of mitochondria [25, 26]. Recent studies in *Drosophila* have shown that a common progenitor gastric stem cell produces the copper, interstitial, and enteroendocrine cells of the stomach [27, 28], and that this progenitor cell is maintained by Wnt signaling [27]. This *Drosophila* model is useful for studying the molecular aspects of gastric stem cells given the flexibility of generating *Drosophila* genetic mutants.

C. elegans is also a useful model for genetic manipulation. The *C. elegans* stomach also bears resemblance to the mammalian stomach in its functional and molecular composition, such as the existence of a vacuolar-type H^+-ATPase genes and proteins [29]. *C. elegans* has also been used to elucidate molecular pathways associated with gastric cancer such as RUNX/CBFβ homologues [30], which provide an accessible framework to genetically model cancer-associated pathways in vivo.

Signaling Pathways in Gastric Cancer

The recent publication of the TCGA data has been important in re-refocusing the typing of human gastric cancers according to genetic alterations rather than by histology. In some instances, now we interpret mouse models of cancer will be reinterpreted. There are no mouse models of gastric cancer if one follows the strict definition of cancer that entails demonstration of cell-autonomous growth and spreading to distant organs (metastasis). Specifically, there are no examples of using soft agar assays, cell line generation, or distant metastatic lesions to verify that the aggressive-appearing lesions found in the mouse stomach and labeled severe dysplasia or carcinoma in situ progress past this stage by demonstrating true malignant capability. However, mouse models remain useful for testing the in vivo role of individual genes either alone or in combination with other loci or environmental

agents. There are models in which hyperplastic, metaplastic, and dysplastic lesions are observed, some with submucosal tissue invasion. The TCGA database only examines the primary cancer and does not compare these genetic changes with those present in metastatic lesions and, those that change after chemotherapy, which can favor the emergence of a variety of malignant new clones from multiple heterogeneous driver and passenger mutations. Moreover, only mouse models are able to track preneoplastic changes from known or hypothetical triggers to better define the timeline of molecular changes. In addition, mouse modeling of specific signaling pathways can provide insight into the relative strength of the mutations with respect to driving the neoplastic process and partnership with synergistic pathways.

Signaling Pathways in the Proximal Versus Distal Stomach

Although it is often difficult to identify the site of the original cancer in humans, gastric cancer is thought to arise in three major sites, the antrum, corpus, and cardia. Since the mouse does not have a clearly defined cardia region, most mouse models of gastric cancer exhibit dysplastic tumors in the gastric body or distal stomach. This result suggests that different regions of the stomach depend on different signaling pathways to drive the hyperplastic phenotype. Therefore the current section will focus on the implications for understanding gastric cancer using rodent models that target major signal transduction pathways. A recent report using a mouse model of IL-1β overexpression from the EBV-L2 promoter, which typically targets squamous mucosa, revealed hyperplastic changes in the first gland of the mouse corpus, which progressed to metaplasia reminiscent of Barrett's esophagus. There was the suggestion that the gland adjacent to the mouse forestomach might mimic the human gastric cardia [31]. Nevertheless, the authors indicated that this approach is a model for esophageal as opposed to gastric cardia cancer [32]. Since the promoter is expressed in squamous cells, the tumor arising in the adjacent columnar epithelium of the first gland likely represents a non-cell autonomous effect.

EGRr/Ras/MapK

K-Ras mutations account for about 10–15 % of the genetic aberrations in gastric cancers [33]. Overexpression of oncogenic K-ras$^{G12D/+}$ driven conditionally from the ubiquitin Ubc9 promoter resulted in depletion of parietal cells (atrophy) and metaplastic changes in the fundic glands [34]. Overexpression of the EGF receptor ligand TGFα produces foveolar hyperplasia reminiscent of Menetrier's Disease [35]. Thus the surface pit cell layer in the corpus appears to be more susceptible to EGFr and *ras* signaling than the antrum.

Notch Signaling

The Notch signaling pathway is one of several cell–cell communication mechanisms initially identified using fly mutagenesis [36]. In mammals, the multiple ligands (Delta, (DLL) 1-4; Delta-like (DLK)1,2 and Jagged (JAG)1, 2) are produced by cells adjacent to the cells that express the receptor (NOTCH1-4). In addition, the pattern of ligand and receptor expression is tissue-dependent. Engagement of the ligand with its receptor initiates proteolysis via a two-step process, which involves ADAM proteases and γ-secretase and ultimately releases the NOTCH intracellular receptor domain (NICD) [37]. This C-terminal NICD migrates to the nucleus where it forms a complex with NOTCH-related transcription factors, e.g., Mastermind (MAML1-3) and RBPJ that subsequently activate canonical target genes HES, HEY, and HEYL [38]. In addition, NOTCH can modulate cell adhesion and NFkβ gene targets through the ability of the NICD to partner with R-Ras and IKKα respectively [37]. Consistent with the tissue specificity of this pathway, it has been reported that NOTCH signaling is oncogenic in gastric and colon cancers, but antineoplastic in squamous cell carcinoma of the esophagus [39]. NOTCH signaling is typically required to maintain the stem cell niche, deep in the crypt zone of the intestine; while a similar function for NOTCH is implied for the gastric antrum due to its similarity to the small bowel in terms of the position and expression of Lgr5+ stem cells at the crypt base [40, 41]. Nevertheless, there are no reports yet demonstrating the effects of modulating this pathway in the glandular stomach.

One possible exception is a mouse model of Barrett's esophagus. Quante et al. overexpressed the IL-1β cytokine in squamous mucosa from the EBV promoter LD2 and observed tumors in the initial gastric gland at the gastroesophageal junction within 12–15 months [31]. In addition, 0.2 % bile acids accelerated the histologic changes, which correlated with increased *Notch* ligand expression. Indeed increased expression of Notch signaling components is consistent with maintenance of the self-renewing stem cell compartment and has been observed in human gastric [42]; esophageal adenocarcinoma [43] and esophageal squamous carcinoma [44]. Thus the Notch signaling pathway clearly contributes to foregut transformation, but overexpression of this pathway has not been performed directly in the gastric epithelium to determine if it is sufficient to drive transformation.

Hedgehog Signaling

Like the discovery of Notch signaling, the Hedgehog pathway in mammals has been associated with cell growth and development as well as neoplastic transformation [45–47]. The three hedgehog ligands (Sonic, Indian, and Desert) are typically expressed and then after release from the epithelium modulate cells in the stroma. Thus normal hedgehog signaling is typically paracrine [48]. Recipient cells express the canonical ligand binding receptor Patched (Ptch1, 2), which normally represses

pathway activation in the absence of ligand via the G-protein coupled receptor Smoothened (Smo) [49]. Upon Smo de-repression, the inactive cytoplasmic glioma-associated transcription factors (Gli 2,3) are released from an inhibitory complex and undergo limited proteolysis revealing positive and negative regulatory domains. Afterwards, the factors translocate to the nucleus to regulate canonical target genes such as Gli1, a third family member of the Gli family and ligand receptors Ptch and Hedgehog Inhibitory Protein (HhIP) [50].

In the stomach, several labs have examined the location and function of sonic hedgehog (Shh) expression. The primary ligand expressed in the stomach is Shh [48, 51]. Although all gastric epithelial cells express Shh, the highest levels in the uninfected stomach occur in parietal cells where it appears to be required for H^+,K^+-ATPase expression and acid production [51–55]. The initial infection of the stomach by *Helicobacter* initiates recruitment of bone marrow-derived immune and mesenchymal cells to the stomach presumably with the intent to initiate repair [56–58]. However, the inflammatory milieu hastens hypochlorhydria within a few months that segues to parietal and zymogenic cell atrophy [51, 59]. Once gastric atrophy sets in as a consequence of the chronic inflammation, hedgehog-dependent immune cells acquire a phenotype sufficient to initiate gastric metaplasia and in some instances dysplasia [60] (Fig. 22.3). Although mouse models do not progress to dysplasia and frank cancer, current studies indicate that the bacterial infection and inflammatory response cooperates with hedgehog signaling to create a micro-environment sufficient for epithelial transformation. Moreover, this conclusion is consistent with prior studies of Hedgehog expression in human gastric cancers and cells lines [61–63].

Wnt/βcatenin

Gastric adenomas and carcinomas in the stomach of FAP patients does not occur frequently [33]. Yet when FAP dependent gastric polyps occur they are associated with pyloric gland and fundic gland polyps [64]. Similarly, examination of mice carrying a truncated APC gene show a predilection for polyps and dysplasia in the antral pyloric glands, suggestion a predisposition of this gastric region to elevated Wnt signaling [65].

Akt/PI3K

There are no direct mouse models of the PhosphoInositol-3 kinase/Akt pathway for gastric cancer. Nevertheless, the pathway appears to be activated in human gastric cancers as a result of chronic EBV infection [20]. Indeed, the initial cloning of the retroviral *v-akt* oncogene in 1987 was initially linked to its cellular homologues AKT1 and AKT2 in primary gastric adenocarcinomas [66]. AKT1

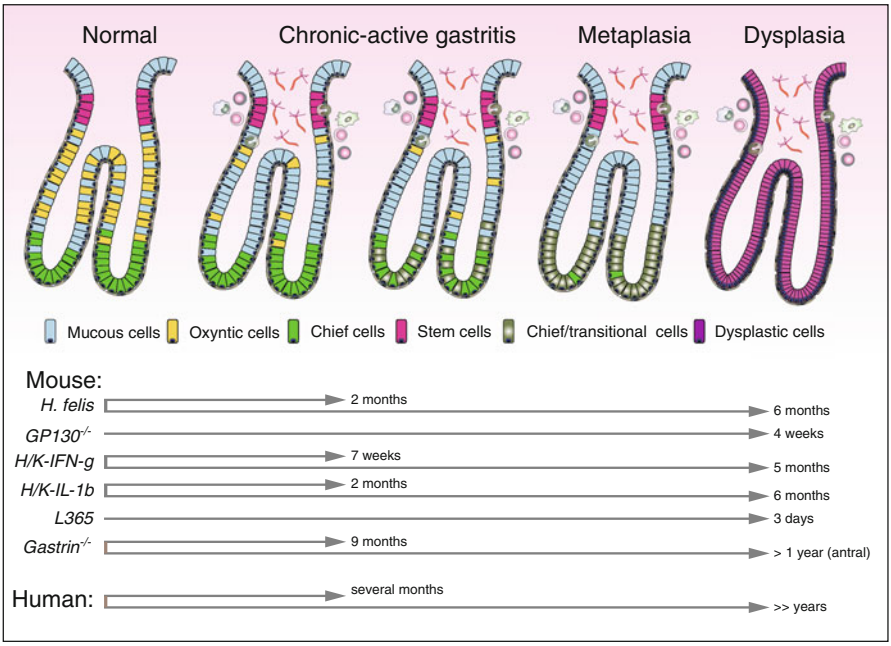

Fig. 22.3 Timeline of chronic gastritis to dysplasia in experimental mouse models. Schematic depiction of *Helicobacter* infection leading to chronic gastritis and ultimately gastric dysplasia. Shown is the two-phase development observable in mice. The first phase indicates chronic-active inflammation after *Helicobacter* infection. The second phase is labeled metaplasia/dysplasia and involves a change in the microenvironment. Dysplasia/cancer in situ is observed in the antrum for Gastrin$^{-/-}$ and GP130$^{F/F}$. Tumors are present in the corpus for the other models. The L-635 model is also shown as a rapid (chemical) model for the induction of SPEM. Note that human subjects develop chronic gastritis over months to years and cancer (CA) over decades

and 2 are primarily cytoplasmic while AKT3 resides in the nucleus [67]. PIK3CA mutations and gene amplification are the two major mechanisms through which the pathway becomes overly active and associated with gastric cancer and poor survival [20, 68]. PI3Ks are found in the nucleus associated with nuclear speckles implicating their role gene expression; while, the oncogene AKT is the canonical effector of PI3K that activates growth pathways, e.g., inhibiting GSK3β [67]. A recent study that examined the role of trefoil factor 1 (TFF1) in gastric tumorigenesis revealed that loss of this factor stabilized β-catenin through reduced GSK3b and Akt phosphorylation [69].

Mouse Models of Gastric Cancer

Rodent models have been used to elucidate details of the molecular mechanisms of various cancers in ways that cannot be ethically studied in humans. It is particularly vital to the study of gastric carcinogenesis, where the host factors, infectious agents

and environment individually and combinatorially influence disease outcome. Although rodents especially mice rarely develop spontaneous gastric cancer, cotton rats (*Sigmodon hispidus*) and *Mastomys natalensis* exhibit a genetic propensity to develop gastric carcinoids [70–72]. Thus, researchers using animal models have focused on the development of chemical, infectious, or genetic tools to experimentally induce gastric cancer in rodents. The paucity of inbred strains of rats, gerbils, and *Mastomys* has limited the use of these rodent models in the study of gastric carcinogenesis. Therefore, most investigators have chosen to use mouse models because of the widespread availability of multiple inbred strains, genetically engineered variants, short breeding cycles, and the accessibility of experimental reagents. A large number of transgenic and knockout mouse models of gastric cancer have been developed using genetic engineering (Table 22.1). A combination of carcinogens and genetic manipulation has been applied to facilitate development of advanced gastric cancer. Therefore, we have focused primarily on the current mouse models of gastric carcinogenesis by comparing their pathological phenotype, experimental limitations, and applications to improve our understanding of the neoplastic process in the stomach. A schematic overview chemical, infectious, and dietary manipulation of the mouse models to generate gastric cancer is depicted in Fig. 22.4.

Table 22.1 Overview murine gastric cancer models

Model	Incidence, %	Duration or age of onset	Location	Phenotype	References
MNU	18–60	50 Weeks	Antrum	Adenocarcinoma, dysplasia	[77, 198]
MNU + *H.pylori*	80	50 Weeks	Antrum	Adenocarcinoma, dysplasia, metaplasia, atrophy	[77, 199]
H.felis	80	15 Months	Corpus	Adenocarcinoma, dysplasia, metaplasia, atrophy	[104, 105]
MNU + *H.felis*	100	36 Weeks	Antrum	Adenocarcinoma, dysplasia, metaplasia, atrophy	[200]
MNU + high salt	50	40 Weeks	Antrum	Adenocarcinoma	[88]
MNU + *H. felis* + high salt	100	40 Weeks	Antrum	Adenocarcinoma	[88]
DMP-777	100	7–14 Days	Corpus	Rapid loss of parietal cells, atrophy, SPEM	[191, 192]
L635	100	7 Days	Corpus	Rapid loss of parietal cells, atrophy, high proliferative SPEM	[193]
Tamoxifen	100	3 Days	Corpus	Rapid, reversible atrophy and metaplasia	[201]

(continued)

Table 22.1 (continued)

Model	Incidence, %	Duration or age of onset	Location	Phenotype	References
INS-GAS	75	20 Months (7 months w/*H.felis*)	Corpus	Adenocarcinoma, dysplasia, metaplasia, atrophy, accelerated by *H.felis*	[127, 128, 202]
GAS$^{-/-}$	60	12 Months	Antrum	Dysplasia, metaplasia, atrophy	[136]
TFF1$^{-/-}$	30	5 Months	Antrum	Multifocal intraepithelial or intramucosal carcinomas	[145]
Gp130$^{F/F}$	100	20 Weeks	Antrum	Atrophy, IM, and SPEM, dysplasia and submucosal invasion	[149]
Atp4a$^{-/-}$	100	12 Months	Corpus	Progressive hyperplasia, mucocystic and incomplete IM	[153]
Potassium channel	100	3 Months	Corpus	Mucous neck cell hyperplasia	[154]
COX-2 + MNU	48	50 Weeks	Antrum	Atrophy, IM and carcinoma	[159]
COX-2 (K19-C2mE)	100	48 Weeks	Corpus	Metaplasia, hyperplasia and tumor	[161]
K-ras (K19-K-ras-V12)	100	3–20 Months	Corpus	3 Months: mucus metaplasia <20 Months: dysplasia and carcinoma	[165, 166]
K-ras (ubiquitous)	100	18 Days	Junction of forestomach and glandular stomach	Rapid loss of parietal cell, hyperplasia, IM	[34]
P27$^{-/-}$ + *H.pylori*	60	60 Weeks	Corpus	IM, Intraepithelial neoplasia and Polypoid adenomas, and in situ or intramucosal carcinoma	[115, 168]
Tgfβ1$^{-/C33S}$	40	16–19 Weeks	Stomach and rectal-anal	Well differentiated invasive adenocarcinoma	[176]

(continued)

Table 22.1 (continued)

Model	Incidence, %	Duration or age of onset	Location	Phenotype	References
TGF-βr II (pS2-dnRII) + *H. pylori*	ND	36 Weeks	Corpus	Adenocarcinoma	[177]
Smad3$^{-/-}$	100	10 Months	Corpus	Metaplasia and Invasive tumor	[178]
Smad4$^{+/-}$	100	>12 Months	Corpus and antrum	Polyposis, hyperplasia, dysplasia, in situ and invasive carcinoma	[180]
RUNX3$^{-/-}$	70	52 Weeks	Corpus and antrum	Adenocarcinoma, IM, SPEM, dysplasia, loss of chief cells	[203]
MT-TGFα	ND	4–6 Weeks	Corpus	Foveolar hyperplasia, loss of parietal cell and chief cell	[204, 205]
TxA23	88	12 Months	Corpus	Oxyntic atrophy, hyperplasia, SPEM, dysplasia, intraepithelial neoplasias	[185]
H/K-ATPase/ hIL-1β	>70	>12 Months	Corpus	Well-differentiated adenocarcinoma, dysplasia, metaplasia, atrophy, increased MDSCs	[189]
H/K-ATPase/ hIL-1β + *H.felis*	10	12 Months	Corpus	Invasive adenocarcinoma	[189]
Myd88$^{-/-}$ + *H. felis*	100	25 Weeks	Corpus	Atrophy, IM, dysplasia	[206]
Menin$^{FL/FL}$;Villin-Cre	11	12 Months	Antrum	Antral tumors	[207]
MTH1$^{-/-}$	14	18 Months	Antrum	Adenomatous hyperplasia, adenoma, or adenocarcinoma	[208]
Atp4b^{cre}; Cdh1$^{FL/FL}$/p53$^{FL/FL}$	69	12 Months	Corpus	Invasive cancer, lymph node metastasis (40 %)	[209]
Atp4b/SV40	100	12 Months	Corpus	Cancer with lymphatic-vascular invasion, lymph node and hepatic metastasis	[210]

Intestinal metaplasia, *SPEM* spasmolytic polypeptide-expressing metaplasia, *MDSCs* myeloid-derived suppressor cells

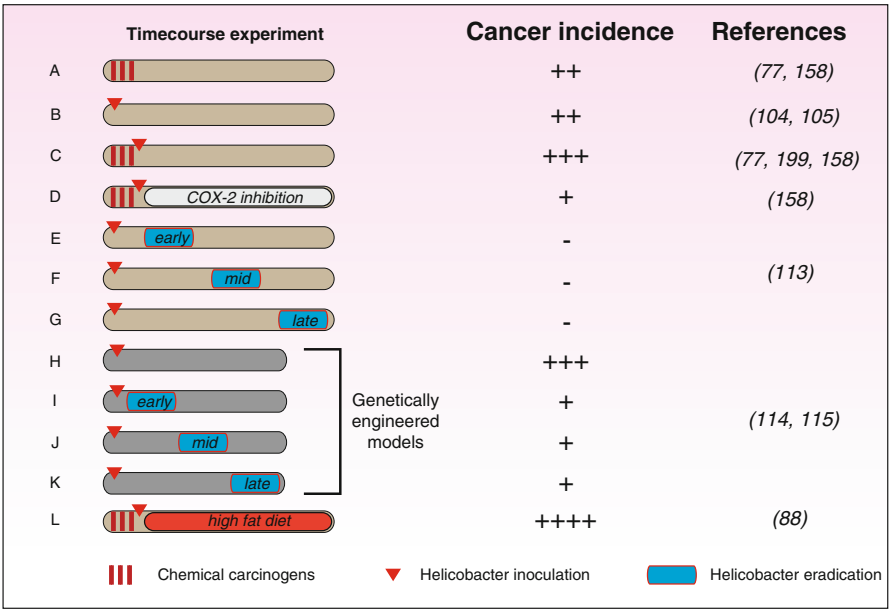

Fig. 22.4 Modifying factors for mouse models of gastric carcinogenesis. Drinking water containing chemical carcinogens MNU (**a**) or *H. felis* inoculation (**b**) strongly enhances stomach carcinogenesis in combination (**c**). Long-term administration of a COX-2 inhibitor (nimesulide) shows strong chemopreventive action against H. pylori-associated gastric transformation (**d**). Early, middle, or late eradication of *H. felis* reduces risk of gastric carcinogenesis in mice (**e**, **f**, **g**). Similar increased risk is observed in INS-GAS or $p27^{-/-}$ mice (**h**–**k**). A high-salt diet further increases the incidence of gastric cancer (**l**)

Chemical Carcinogen-Induced Models of Gastric Cancer

N-Nitroso Compounds (MNNG and MNU)

Prior to the widespread acceptance of *Helicobacter* infection, researchers tested the utility of several chemical carcinogens such as benzo-a-pyrene,3-methylcholanthrene and 2-acetyl aminofluorene in animals starting in the 1930s, but the incidences of chemically induced stomach cancer were low. In 1967, Sugimura and Fujimura were able to report higher yields of adenocarcinomas in the glandular stomachs of rats treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [73]. Under acidic conditions, MNNG is converted to *N*-methyl-*N'*-nitroguanidine, which is capable of alkylating the purine bases of DNA, RNA, and some amino acids leading to subsequent mutations. Moreover, MNNG was found to be a very potent gastric carcinogen in Mongolian gerbils. Treatment with 400 ppm of MNNG in drinking water for 50 weeks resulted in 64 % gastric adenocarcinomas in the gerbils. However, the mouse glandular stomach was found to be relatively resistant to MNNG. Administration of MNNG in the drinking water of rats over their life span resulted in adenomatous tumors only in the glandular epithelium of the stomach [74].

The utility of another N-nitroso compound, *N*-methyl-*N*-nitrosourea (MNU) as a gastric carcinogen was tested in mice and found to be more efffective. Weekly gavage with 0.5 mg MNU in the Balb/c mice resulted in premature death due to squamous cell carcinoma in the forestomach. Surgical removal of the forestomach prior to MNU treatment leads to 100 % development of glandular cancer by 40 weeks [75]. Thus, the glandular stomach is sensitive to the MNU, but the greater sensitivity of the forestomach to MNU obscured the phenotype. Further testing by Tatematsu et al. revealed that low dose (30–120 ppm) administration in the drinking water proved more effective in targeting the glandular stomach without tumors in the forestomach [76]. The same group further demonstrated that the induction efficiency of adenocarcinomas in the glandular stomach depended on MNU concentration rather than total quantity. As a result, they established a protocol of 240 ppm MNU in the drinking water biweekly for 5 weeks as the standard method to induce gastric carcinogenesis in mice [77]. This MNU mouse model opened up new approaches for using transgenic and knockout mice to investigate various signaling pathways or transcription factors in gastric carcinogenesis. It should be noted that MNNG- and MNU-induced tumors primarily in the antral mucosa and rarely in the normal fundic mucosa, like the tumor types found in humans [1].

Dietary Salt

A high salt intake has been implicated in a number of human case-control and ecological studies from various geographical regions as a risk factor for stomach cancer [78, 79]. This phenomenon has been addressed in rodents by assessing the effects of sodium chloride administration. Sodium chloride possibly decreases the viscosity of gastric mucins and might reduce the protective mucous barrier. Acute exposure of rats to a single dose of hypertonic sodium chloride immediately damages the surface mucous cell layer and then stimulates regenerative cell proliferation returning the mucosa to homeostasis within 24–48 h after the exposure [80, 81].

The effect of chronic sodium chloride administration in high-salt diets has been evaluated in a number of rodent studies. When given alone, a high-salt diet causes atrophic gastritis in gerbils [82] and C57BL/6 mice [83, 84], but no evidence of tumors. When administered with MNNG or Nitroquinolone-1-oxide (NQO), sodium chloride promotes stomach carcinogenesis in the rats [85, 86] in a dose-dependent manner [87]. A high-salt diet also enhances the multiplicity of gastric tumors in MNU-treated mice and synergizes with the transforming effects of a *Helicobacter* infection [88]. Therefore the available data from experimental rodent models clearly supports the concept that high salt intake alone does not induce but rather increases the risk for gastric neoplasia.

Other Environment-Related Agents

Several other environment-related agents thought to promote the onset of gastric carcinogenesis have also been tested in mouse models. Administration of catechol (a phenol in cigarette smoke, perfumes, and insecticides) in the diet has been shown

to enhance preneoplastic and neoplastic lesions in the Balb/c mouse glandular stomach with MNU treatment in a dose-dependent manner [89]. The same group also demonstrated that low dose catechol at predicted human exposure levels has a limited effect on MNU-induced cancers [90]. However, it should be noted that 0.8 % catechol alone is sufficient to induce adenocarcinomas in the rat stomach. However the incidence varied with the rat strain suggesting that genetic background influences susceptibility to catechol carcinogenicity [91, 92].

Butylated hydroxyanisole (BHA), an antioxidant commonly used in food preservatives was fed at a concentrate of 0.5–2.0 % to Fisher 344 rats, Syrian golden hamsters, and B6C3F1 mice for 2 years, and caused increased forestomach hyperplasia and papillomas in all three species. Nevertheless, forestomach (squamous cell) carcinoma was only observed in rats and hamsters underscoring that variations in the genome modulate carcinogen susceptibility [93–95].

Ethylene dibromide (EDB) a soil, grain fumigant, chemical intermediate, and solvent increased forestomach cancer when administered to rats and B6C3F1 mice by gavage but not when given by inhalation [96].

Bacterial Models

Helicobacter

Helicobacter is thought to account for about 80 % of gastric cancers. Although many animals have been successfully infected with human *H. pylori*, none of these early models proved sufficiently similar to the situation with human *H. pylori* infection and pathology. Then in 1998, Watanabe et al. published the first successful experiment of gastric cancer induced by *H. pylori*. After 62 weeks of infection, 10 of 27 (37 %) infected Mongolian gerbils developed gastric tumors with histological similarity to human intestinal-type gastric cancer. In general mice, especially the C57BL/6 strain, were remarkably resistant to colonization with various *H. pylori* strains [97, 98] until Lee et al. successfully adapted a clinical Cag A and Vac A-expressing strain called SS1 (Sydney strain) that efficiently colonized the mouse stomach [99]. High levels of colonization have been achieved in C57BL/6, while colonization levels in Balb/c, DBA/2, and C3H/HeJ strains were lower. Although the SS1 strain causes chronic active gastritis and atrophy after 8 months of infection, it does not induce gastric carcinoma in C57BL/6 wild type mice even after 2 years [100].

Thus, alternative mouse models of gastric *Helicobacter* infection were explored. In 1990, *Helicobacter felis*, a close relative of *H. pylori* was isolated from the cat stomach and shown to efficiently colonize the mouse stomach causing more severe gastritis than that induced by *H. pylori* [101–103]. *H. felis*-infected mice show gastric metaplasia, dysplasia, and eventually progress to invasive cancer after extended periods of infection [104, 105]. However despite extensive submucosal cystic lesions observed in the *H. felis*-infected mice, no metastasis was reported, nor were other means of assessing malignant potential performed, suggesting that the model still falls short of mimicking true carcinoma. In fact a surprising finding was the

observation that infecting mice heterozygous for p53, mitigated the appearance of invasive lesions. This result seems counter-intuitive given the recent observation that subjects with p53 mutant gastric cancer have worse survival [22].

Following *H. felis* or *H. pylori* infection, the immune response in the C57BL/6 strain is predominantly Th1-skewed with low bacterial loads and high levels of epithelial cell damage whereas, the Th2-predominant Balb/c strain exhibits higher bacterial load and less evidence of cell damage [99, 103, 104, 106]. Both *H. pylori*-infected C57BL/6 and Balb/c mice show a marked influx of mononuclear cells [103, 107].

Much of the research now focuses on the cancer-preventive effect of *H. pylori* eradication. Several cohort studies and randomized controlled trials have shown that *H. pylori* eradication can halt the histological progression from chronic gastritis to gastric adenocarcinoma and even induce regression of atrophy in patients with tumor-associated infection [108–112]. However, the effect of most of these interventions is less evident. The striking observation was that those gastric cancers that occurred after eradication treatment were confined to those subjects who already had atrophic gastritis and intestinal metaplasia at baseline. It suggests that there may be a "point of no return" beyond which the precancerous cascade can no longer be reversed. Antimicrobial treatment studies have been conducted in mouse models and might help us address the uncertain questions in this field. In *H. felis*-infected C57BL/6 mice, eradication of *Helicobacter* at early (2 months post infection) or at later (6 months) intervals led to regression of inflammation, restoration of parietal cell mass, and reestablishment of normal architecture. Late eradication (1 year) restricted the progression to dysplasia [113]. In *H. pylori*-infected hypergastrinemic INS-GAS mice treated with antibiotics, the progression of gastric lesions after curative treatment for *H. pylori* was significantly less than without eradication [114]. In $p27^{-/-}$ mice, *H. pylori* eradication during the early (15 weeks post infection) and late (45 weeks) periods of infection effectively reduced development of gastric transformation even though mice had already showed pseudopyloric metaplasia. These studies suggest that *Helicobacter* eradication might be beneficial for gastric cancer prevention in humans even when given relatively late in the natural history of the disease [115].

Helicobacter *Coinfection with Other Microorganisms*

In C57BL/6 models in which immune response was shifted toward a Th2-polarized response by coinfection with an intestinal helminth attenuated *Helicobacter*-dependent atrophy and metaplasia without reducing inflammation, which suggested that the epithelial changes were not directly related to the severity of the inflammatory response [116]. A recent study showed that helminth infection reduces *H. pylori*-induced gastric lesions while inhibiting changes in gastric flora [117]. Conversely, shifting the immune cytokine profile of resistant host Balb/c mice toward a Th1-polarized response by prior infection with the Th1-provoking protozoan *Toxoplasma gondii* confers susceptibility to chronic active gastritis and dysplastic lesions after *H. felis* infection [118]. These two models offer a potential

explanation for the "African enigma," which infers a high *H. pylori* prevalence with relatively low gastric cancer burden, especially in countries with frequent endogenous parasitic diseases [119, 120].

Epstein-Barr Virus

It is likely that some cases of gastric cancer might be attributable to other infectious agents. For example, Epstein-Barr virus (EBV) has been linked to 6–16 % of gastric cancer cases worldwide [121–123]. EBV-associated gastric cancer (EBV-GC) has unique morphologic and phenotypic features, and might also differ considerably from EBV-negative gastric cancers [124]. However, research regarding the role of EBV in gastric carcinoma has been hampered by the absence of a suitable model system. To investigate the mechanism of EBV-induced gastric cancer (EBV-GC), researchers have explored models of EBV engraftment using infected epithelial cell lines. SNU-719 is a gastric carcinoma cell line established from a Korean patient that shows modified latency of EBV infection closely resembling EBV-GC [125]. After subcutaneous injection of the SNU-719 gastric cancer cell line into athymic nude mice (BALB/c nu/nu) with the Matrigel substrate as an irritant, all mice developed tumors, which showed characteristics of moderately differentiated carcinoma with no gland formation and areas of necrosis [124].

Genetically Engineered Mouse Models (GEMMs)

Transgenic mouse models have proved to be the most powerful tool for dissecting the importance of individual host susceptibility genes and signaling pathways. These have included abnormal expression of growth factors and cytokines, as well as mutations in oncogene and tumor suppressor gene loci. Most of these models were developed on the *C57BL/6* genetic background. Representative highlights of the use of mouse models is shown in Fig. 22.3 with a more detailed list of mouse models listed in Table 22.1.

Gastrin Mutants

Gastrin is a crucial peptide hormone released by G cells located in the antrum that stimulates secretion of gastric acid (HCl) by the parietal cells and aids in gastric motility. Altered gastrin gene expression and secretion leads to disturbances in gastric epithelial cell dynamics potentially promoting gastric cancer, as revealed by the various mouse models described below.

INS-GAS Mice

Given the known properties of gastrin as a mucosal growth factor, hypergastrinemia was postulated to be a factor promoting the development of gastric cancer. The insulin-gastrin (INS-GAS) mice were engineered as a transgene in the FVB/N strain, which overexpress the human gastrin gene under the control of the mouse insulin promoter [16, 126]. These mice have elevated serum levels of human amidated gastrin (sustained hypergastrinemia) and spontaneously develop gastric atrophy, metaplasia, dysplasia, and eventually progress to invasive gastric tumors in the corpus (submucosal cysts) by 20 months of age without lymph node invasion or distant metastasis [16, 127, 128]. Amidated gastrin in the corpus up-regulates growth factors [16] in combination with induction of apoptosis in gastric epithelial cells, particularly parietal cells [129], both of which may trigger Correa's cascade and lead to gastric cancer. Due to its lower threshold for carcinogenesis, the INS-GAS mouse has proven to be a valuable model of gastric cancer development when used in combination with other agents. Infection of INS-GAS mice with *H. felis* or *H. pylori* led to accelerated carcinogenesis (7 months after infection) [16, 130], and more severe lesions were observed in the male INS-GAS mice [130]. However tumor development was delayed for months in gnotobiotic INS-GAS mice mono-infected with *H. pylori* compared to INS-GAS mice colonized with *H. pylori* and complex enteric microbiota [131]. A most recent study demonstrated that gnotobiotic INS-GAS colonized with *H. pylori* and three bacterial members of Altered Schaedler Flora, developed gastritis and premalignant gastric lesions equivalent to *H. pylori*-infected INS-GAS mice with complex microflora [132]. These data support the notion that hypergastrinemia and *Helicobacter*-induced tumors require additional microflora. The metaplasia induced in the INS-GAS mouse also involve reactivation of the Hedgehog pathway [133] whereas, inhibition of the gastrin/CCK2 and histamine H2 receptor limits the development of gastric neoplasia in these mice [134].

Gastrin-Deficient Mice

Gastrin-deficient mice ($Gast^{-/-}$) on a mixed C57BL6/129Sv background are hypochlorhydric and develop spontaneous gastric antral tumors at 12 months of age [135, 136]. Tumors in this mouse model are associated with bacterial overgrowth [137] and inflammation [136, 138]. We reported multiple inflammatory mediators, such as IL-1β, IL-11, and the Tgfβ pathway components activin A and follistatin with epithelial Gli2 appear to be important epithelial drivers of the histologic changes during antral transformation in the $Gast^{-/-}$ mice [139, 140]. Takashi et al. investigated the role of gastrin in *H. felis*-infected hypergastrinemic transgenic (INS-GAS) mice, GAS-KO mice, and C57BL/6 wild-type mice on a uniform C57BL/6 genetic background housed under specific-pathogen-free conditions [141]. Their results showed that gastrin has a distinct effect on the gastric corpus

and antrum in the setting of chronic gastric *Helicobacter* infection. While gastrin is possibly an essential cofactor for gastric corpus carcinogenesis, gastrin deficiency can predispose animals to antral tumorigenesis, and thus any imbalances in gastrin physiology may represent a risk for gastric transformation.

The Trefoil Factor 1 (Tff1) and gp130 Mutants

The tumor suppressor Trefoil factor 1 (TFF1) protein is normally expressed by the surface pit cells and is abnormally expressed in gastrointestinal diseases and various cancers [142–144]. *Tff1*$^{-/-}$ mice on a 129/Svj mixed genetic background develop antropyloric adenomas and 30 % develop multifocal intraepithelial or intramucosal carcinomas [145]. A recent study has shown that loss of *Tff1* leads to activation of β-catenin signaling and gastric tumorigenesis through induction of PP2A, a major regulator of AKT-GSK3β signaling [69]. Genetic deletion of *cyclo-oxygenase-2* (*Cox-2*) in the *Tff1*$^{-/-}$ mice resulted in reduced adenoma size and ulceration with a chronic inflammatory reaction at the site of the adenoma. Moreover, selective inhibition of *Cox-2* resulted in regression of established gastric adenomas in *Tff1*$^{-/-}$ mice [146].

Tff1 expression is strongly suppressed in gp130-mutant mice (*gp130*$^{F/F}$). Interestingly, the phenotype of gp130$^{F/F}$ mice in many ways mimics that of *TFF1*$^{-/-}$ and Gast$^{-/-}$ mice. Glycoprotein 130 (gp130) is a ubiquitously expressed, signal-transducing receptor that forms part of the receptor complex for the interleukin-6 (IL-6) family of cytokines. IL-6 and IL-11 are the dominant IL-6 family cytokines in the gastric mucosa, and the only cytokines of the family that exclusively utilize gp130 homodimers. Homeostatic gp130 signaling following receptor activation can trigger three alternate signaling cascades: the JAK/STAT, SHP-2/ERK/MAPK, or Src/PI3/AKT pathway. Under homeostatic conditions, these three gp130 pathways are tightly controlled by multiple negative feedback mechanisms [147]. *Gp130*$^{F/F}$ mice generated by a knock-in point mutation that converts a tyrosine (Y) to a phenylalanine (F) blocking phosphorylation at the receptor site recognized by the SHP-2/SOCS3 signaling complex [148]. The mutant receptor was generated to examine the role of gp130 ligands and their signaling pathways in hematopoiesis and inflammation. The knock-in mutation prevents SHP-2 from docking and ablates signal transduction through the SHP2/ERK/MAPK cascade. The absence of one of the three signaling pathways transduced by the gp130 receptor subsequently creates a signal imbalance which favors the JAK/STAT1/3 pathway in the absence of negative feedback from SHP2. A principle feature of gp130$^{F/F}$ mice is the phenotypic change in the distal stomach of these mice characteristic of human gastric adenocarcinoma, including rapid development of gastritis, atrophy, intestinal metaplasia and SPEM, dysplasia and submucosal invasion by 20 weeks of age [149], making them an excellent model to study gastric cancer progression. In a mouse model with compound gp130$^{F/F}$/STAT3$^{+/-}$ mutants, mice have significantly smaller tumors and reduced gastric inflammation, proinflammatory cytokines, and

chemokines. Chronic treatment of the gp130$^{F/F}$ mice with antibiotics reduced tumor mass by reducing activated macrophages in the gastric mucosa [150]. Moreover on the gp130$^{F/F}$ background, neither *Rag1*$^{-/-}$ mice, which lacks T, B, and NKT cells, nor the *Perforin*$^{-/-}$ mice, which have diminished cytotoxic T cell function, reduce tumor development [151], suggesting that macrophages might play an aggressive tumor-promoting role.

Parietal Cell Mutants

Parietal cells in the fundus or corpus stomach secret hydrochloric acid (HCl) to maintain a highly acidic environment and promote the activation of stomach enzymes for digestion such as pepsin. Three different signaling pathways—a bioamine (histamine), a neurotransmitter (acetylcholine), and a hormone (gastrin) regulates parietal cell acid secretion. Loss of parietal cells or their ability to secrete acid predisposes the gastric epithelium to metaplasia and cancer.

The enzyme hydrogen potassium ATPase (H^+,K^+-ATPase) is unique to the parietal cell and is the most critical component of the ion transport system mediating acid secretion in the stomach. The enzyme consists of two subunits, a 114-kDa α-subunit (Atp4a) and a 35-kDa (protein moiety) β-subunit (Atp4b). Mice homozygous null for the α-subunit (*Atp4a*$^{-/-}$) alleles exhibit normal systemic electrolyte and acid-base status but are achlorhydric and hypergastrinemic [152]. Chronic achlorhydria and hypergastrinemia in aged *Atp4a*$^{-/-}$ mice produced progressive hyperplasia, mucocystic and incomplete intestinal metaplasia, and induction of growth factors without histological evidence of neoplasia [153].

The potassium channel is crucial for H^+,K^+-ATPase activity. The KvLQT1 gene encodes a voltage-gated potassium channel. KvLQT1 knockout mice display a threefold enlargement of the stomach resulting from mucous neck cell hyperplasia (SPEM) by 3 months of age [154].

Histamine H2 receptor (H2R) is expressed on parietal cells and functions to stimulate gastric acid secretion. The H2R-deficient mice exhibit hypergastrinemia and marked hypertrophy due to an increase in the numbers of parietal cells, ECL cells, and other types of cells. It should be noted that the morphological characteristics of the parietal cells were remarkably altered in H2R-deficient mice. The size of parietal cells in these mice was significantly smaller despite increased cells numbers [155].

Oncogene and Tumor Suppressor Gene Mutants

COX-2

Overexpression of cyclooxygenase 2 (COX-2) is involved in gastric cancer and is highly induced in *H. pylori* infection. There are compelling epidemiological data to suggest that long-term use of nonsteroidal anti-inflammatory drugs is associated

with a significant reduction in gastric cancer risk, largely attributed to the inhibition of COX-2 enzymes [156–158]. COX-2 transgenic mice, generated on a C57BL/6 genetic background expressing full-length human COX-2 cDNA, showed an increased frequency of MNU-induced gastric cancer [159]. Treatment with celecoxib, a specific COX-2 inhibitor, prevents MNNG-induced gastric cancer in a rodent model [160]. Transgenic mice (*K19-C2mE*) simultaneously expressing *COX-2* and the microsomal *prostaglandin E synthase (mPGES)-1* develop metaplasia, hyperplasia, and tumorous growths at 48 weeks with heavy macrophage infiltrations after *Helicobacter* infection [161], through a tumor necrosis factor-α(TNF-α) dependent pathway [162]. Treatment of K19-C2mE mice with NS-398, a COX-2 selective inhibitor, for 4 weeks completely suppressed gastric hypertrophy, reducing mucosal thickness to that found in the age-matched wild type. These results clearly indicate that increased levels of COX-2 are essential for the gastric pathology in K19-C2mE mice [161]. Thus, COX-2 is a prime target for chemoprevention of stomach cancer.

K-ras

One oncogene that has been strongly linked to the development of chronic inflammation and a variety of human cancers has been *K-ras*. Activating K-*ras* mutations are found in approximately 5–20 % of gastric cancers [163], and are more prevalent in intestinal-type gastric cancers [164]. A transgenic model (K19-K-ras-V12) in which the cytokeratin 19 (K19) promoter targets *K-ras-V12* mutant gene expression to the gastric mucus neck cells was used to analyze the function of K-ras on the stomach carcinogenesis [165]. Activated K-ras in this context increased recruitment of bone marrow-derived inflammatory cells that contribute to the stromal microenvironment and causes gradual parietal cell loss and mucous neck cell hyperplasia, comparable to *H. felis* infection [166]. Introduction of $K\text{-}ras^{G12D}$ mutation controlled by inducible, Cre-mediated recombination in the K19 expressing lineage in another mouse model (*CK19CreERT; LSL-*$Kras^{G12D}$ mice) led to numerous hyperplasias, metaplasias, and adenomas in the stomach as well as in the oral cavity, colon, and lungs [167]. The effects in mice of ubiquitous activation of *K-ras* were determined in a mouse model created by cross *UBC9-CreERT* mice with *LoxP-STOP-LoxP-*$Kras^{G12D}$ mice. Systemic activation of K-ras leads to rapid changes in gastric cellular homeostasis, and resulted in activation of the MAPK pathway and hyperproliferation of squamous epithelium in the forestomach and metaplasia in the glandular stomach, resembling the preneoplastic changes that take place during gastric carcinogenesis in humans [34]. It suggests mutant *K-ras* signaling modulates important molecular events in the initiating gastric carcinogenesis.

p27^{Kip1}

The cyclin-dependent kinase (CDK) inhibitor p27^{Kip1} has an important role in cell cycle regulation and is associated with many malignancies, including gastric cancer [168]. *Helicobacter* infection is associated with complete loss of p27^{Kip1} or cytoplasmic p27^{Kip1} retention [169, 170]. p27^{Kip1} mislocalization to the cytoplasm blocks its ability to suppress nuclear cell cycle events. *p27^{Kip1}* knockout mice develop mild gastric hyperplasia, random foci of moderate metaplasia and atypia or low-grade dysplasia. After *H. pylori* infection, these mice show intestinal metaplasia, high-grade gastric intraepithelial neoplasia, and polypoid adenomas and, in some cases, in situ or intramucosal carcinoma, all of which were more advanced than in WT mice [168]. Thus, the *p27^{Kip1}*-deficient mouse is a useful model to examine the pathogenesis of *H. pylori* in gastric carcinogenesis and to test eradication and chemopreventive strategies [115].

Inflammation Mediators and Cytokine Mutants

Inflammatory mediators and cytokines are vital for developing a tumor microenvironment, which further stimulate tumor progression. The following GEMMs have been useful for separating events due to an overactive immune system versus parietal cell atrophy.

TGF-β Signaling

Transforming growth factor beta (TGF-β) is a cytokine that controls proliferation, cellular differentiation, and other functions in most cells. TGF-β exists as three isoforms designated TGF-β1, TGF-β2, and TGF-β3. All three isoforms bind to the TGF-β receptor II that recruits and phosphorylates TGF-β receptor I. The TGF-β1 signaling pathway is commonly altered in gastric cancer [171–173]. TGF-β1 null mice develop a severe wasting syndrome from inflammatory cell infiltration into various tissues including the stomach [174], which eventually exhibits gastric epithelial hyperplasia and SPEM, contributing to the early lethality [175]. To circumvent early demise, Ota et al. generated mice encoding a TGF-β1 mutant that prevents ligand binding to the latent TGF-β binding protein (*Tgfb1$^{-/C33S}$*) [176]. About 55 % of these mice survived at 12 weeks and displayed multi-organ inflammation and an elevated incidence of various types of gastrointestinal solid tumors. A *Tgfb1$^{-/C33S}$; Rag2$^{-/-}$* chimeric mouse line that lacks mature lymphocytes was generated to further investigate the relative contribution of TGF-β1 to lymphocyte-mediated inflammation in gastrointestinal tumorigenesis. No tumors were found in the stomachs of these mice, demonstrating that active TGF-β1 enhanced the levels of

lymphocyte-dependent inflammation and gastric epithelial proliferation, while blocking TGFβ-mediated immune cells can impede tumor development [176].

A dominant-negative transgenic model (pS2-dnRII) of the TGF-β receptor II was expressed under the control of the TFF1 promoter to restrict expression of the transgene to the stomach. These mice showed a higher proliferation index and a higher incidence of gastric adenocarcinoma after *H. pylori* infection [177]. SMAD proteins are downstream effectors of the TGF-β signaling pathway. Smad3-null mice develop gastric tumors in the fundus initiated from the forestomach/glandular transition zone along the lesser curvature [178]. Similarly, heterozygous Smad4 knockout mice develop gastric cancer spontaneously [179, 180]. However, selective loss of Smad4-dependent signaling in T cells leads to spontaneous epithelial cancers throughout the gastrointestinal tract in mice, with induction of abundant Th2 and Th17 type cytokines, suggesting that Smad4 signaling in T cells is required for suppression of gastrointestinal cancer [181, 182].

Autoimmune Model (TxA23 Mice)

Autoimmune gastritis also triggers a chronic state of gastric inflammation. Individuals with severe autoimmune gastritis exhibit an increased risk of gastric cancer [183, 184]. A model of autoimmune gastritis (TxA23 mice) was developed to investigate how to suppress chronic inflammation in the gastric mucosa. These mice mimic many aspects of the corresponding human condition and develop gastric lesions in accordance with the Correa paradigm [185, 186].

Interleukin-1β

Interleukin-1β (IL1-β) is a pleiotropic proinflammatory cytokine that has profound effects on inflammation and immunity. The polymorphism of IL1-β has been shown to increase the risk of gastric cancer [187, 188]. Transgenic mice with stomach-specific human *IL1-β* expression (*H^+,K^+-ATPase-hIL-1β*) develop spontaneous gastric inflammation and marked gastric hyperplasia, parietal cell loss, metaplasia, and dysplasia, all of which are accelerated by *H. felis* infection. Transgenic overexpression of *IL-1β* in the stomach mobilizes myeloid-derived suppressor cell (MDSC) recruitment at the earliest histopathologic stages of progression from gastric inflammation to cancer. This mouse line was crossed to *$Rag2^{-/-}$* mice to generate lymphocyte deficient *IL-1β* transgenic mice, which displayed the spontaneous development of atrophic gastritis, metaplasia, and dysplasia, accompanied by a marked increase in the number of MDSCs in the stomach, blood, and spleen, suggesting that MDSCs are a critical mediator of early stages of gastric transformation [189]. On the contrary, *IL-1β* null mice show decreased recruitment of neutrophils and macrophages, which suppress the multiplicity of gastric tumors in the setting of *H. pylori* infection [190].

Models of Precancerous Change

In addition to mouse models of gastric cancer, there are a number of models that show precancerous lesions. Most of these models do not progress to neoplasia. Goldenring and coworkers developed two short-term SPEM models by using DMP-777 and L-635, both are chemical protonophores that elicit a rapid loss of parietal cells followed by the emergence of foveolar hyperplasia and SPEM [191, 192]. DMP-777 is a neutrophil elastase inhibitor. As a result, mice treated with DMP-777 for 14 days develop SPEM in the absence of significant inflammation. By contrast, the DMP-777 enantiamer called L-635 is a structurally related β-lactam compound that develops more advanced proliferative SPEM lesions associated with an intestinal metaplastic phenotype and more prominent inflammatory infiltrate in just 3 days of treatment. Thus L-635 rapidly induces the mucosal phenotype associated with 6 (or more) months of *H. felis* infection. These results indicate that the SPEM phenotype per se might be driven primarily by the inflammation than by parietal cell atrophy. Moreover, a recent study showed that M2 macrophages are the critical immune cell driver of this rapidly generated metaplasic change after loss of parietal cells [193]. Thus, strategic use of these two compounds in mice could help attribute the origin of the mucosal effects induced by parietal cell atrophy versus the inflammatory response.

Model of Bone Marrow-Derived Gastric Cancer Stem Cells

The main function of gastric stem cells is to maintain the integrity of the gastrointestinal epithelium and replenish all of the mature cell lineages. Recent advances in gastric stem cell biology have lead to a new paradigm in which chronic inflammation causes tissue injury and local tissue stem cell failure, followed by recruitment and permanent engraftment of circulating bone marrow-derived stem cell (BMDC) in the tissue stem cell niche. In this way, BMDC essentially take over the function of the tissue stem cell in a severely damaged mucosa [194–196]. To test the role of BMDC in tissue repair after treatment with gastric carcinogens, mice were myeloablated via irradiation and then transplanted with gender-mismatched BM. To further facilitate tracking of the BMDC, BMDCs carried a reporter (GFP or β-gal). After recovery of immune function, mice were infected with *H. felis* and after a period time, engraftment of BMDCs into the stomach was detected and found to differentiate into a range of epithelial cells [197]. After 30 weeks of engraftment, antralized glands and metaplastic cells at the squamo-columnar junction were entirely replaced by BMDCs. One year of infection, most mice developed invasive neoplastic glands, which arose from donor marrow cells. However, neither acute ulceration by cryo-injury or acetic acid nor selective but reversible parietal cell ablation required BMDCs for repair and neither condition was associated with any evidence of marrow engraftment into the gastric epithelium [197].

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References

1. Correa P, Haenszel W, Cuello C, Tannenbaum S, Archer M. A model for gastric cancer epidemiology. Lancet. 1975;2:58–60.
2. Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. J Clin Invest. 2007;117:60–9.
3. McPeak E, Warren S. Histologic features of carcinoma of the cardio-esophageal junction and cardia. Am J Pathol. 1948;24:971–1001.
4. Camargo MC, Anderson WF, King JB, Correa P, Thomas CC, Rosenberg PS, Eheman CR, Rabkin CS. Divergent trends for gastric cancer incidence by anatomical subsite in US adults. Gut. 2011;60:1644–9.
5. Dixon MF, Mapstone NP, Neville PM, Moayyedi P, Axon AT. Bile reflux gastritis and intestinal metaplasia at the cardia. Gut. 2002;51:351–5.
6. Nam KT, Lee HJ, Sousa JF, Weis VG, O'Neal RL, Finke PE, Romero-Gallo J, Shi G, Mills JC, Peek Jr RM, et al. Mature chief cells are cryptic progenitors for metaplasia in the stomach. Gastroenterology. 2010;139:2028–37. e2029.
7. Goldenring JR, Nam KT, Wang TC, Mills JC, Wright NA. Spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia: time for reevaluation of metaplasias and the origins of gastric cancer. Gastroenterology. 2010;138:2207–10. 2210 e2201.
8. Gonzalez CA, Sanz-Anquela JM, Gisbert JP, Correa P. Utility of subtyping intestinal metaplasia as marker of gastric cancer risk. A review of the evidence. Int J Cancer. 2013;133:1023–32.
9. Rugge M, Capelle LG, Fassan M. Individual risk stratification of gastric cancer: evolving concepts and their impact on clinical practice. Best Pract Res Clin Gastroenterol. 2014;28:1043–53.
10. Stange DE, Koo BK, Huch M, Sibbel G, Basak O, Lyubimova A, Kujala P, Bartfeld S, Koster J, Geahlen JH, et al. Differentiated Troy+ chief cells act as reserve stem cells to generate all lineages of the stomach epithelium. Cell. 2013;155:357–68.
11. Chen D, Aihara T, Zhao CM, Hakanson R, Okabe S. Differentiation of the gastric mucosa. I. Role of histamine in control of function and integrity of oxyntic mucosa: understanding gastric physiology through disruption of targeted genes. Am J Physiol Gastrointest Liver Physiol. 2006;291:G539–44.
12. Walsh JH. Gastrointestinal Hormones. In: Johnson LR, Alpers DH, Christensen J, Jacobson ED, Walsh JH, editors. Physiology of the gastrointestinal tract. New York: Raven; 1994. p. 1–128.
13. Bachwich D, Merchant J, Brand SJ. Identification of a cis-regulatory element mediating somatostatin inhibition of epidermal growth factor-stimulated gastrin gene transcription. Mol Endocrinol. 1992;6:1175–84.
14. Dockray GJ, Varro A, Dimaline R. Gastric endocrine cells: gene expression, processing, and targeting of active products. Physiol Rev. 1996;76:767–98.
15. Saqui-Salces M, Dowdle WE, Reiter JF, Merchant JL. A high-fat diet regulates gastrin and acid secretion through primary cilia. FASEB J. 2012;26:3127–39.
16. Wang TC, Dangler CA, Chen D, Goldenring JR, Koh T, Raychowdhury R, Coffey RJ, Ito S, Varro A, Dockray GJ, et al. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. Gastroenterology. 2000;118:36–47.
17. Stolte M. Helicobacter pylori gastritis and gastric MALT-lymphoma. Lancet. 1992;339:745–6.
18. Kidd M, Gustafsson B, Modlin IM. Gastric carcinoids (neuroendocrine neoplasms). Gastroenterol Clin North Am. 2013;42:381–97.
19. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. an attempt at a histo-clinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
20. Network TCGAR. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.

21. Wang K, Yuen ST, Xu J, Lee SP, Yan HH, Shi ST, Siu HC, Deng S, Chu KM, Law S, et al. Whole-genome sequencing and comprehensive molecular profiling identify new driver mutations in gastric cancer. Nat Genet. 2014;46:573–82.
22. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21:449–56.
23. McNulty M, Puljung M, Jefford G, Dubreuil RR. Evidence that a copper-metallothionein complex is responsible for fluorescence in acid-secreting cells of the Drosophila stomach. Cell Tissue Res. 2001;304:383–9.
24. Filshie BK, Poulson DF, Waterhouse DF. Ultrastructure of the copper-accumulating region of the Drosophila larval midgut. Tissue Cell. 1971;3:77–102.
25. Dubreuil RR, Grushko T, Baumann O. Differential effects of a labial mutation on the development, structure, and function of stomach acid-secreting cells in Drosophila melanogaster larvae and adults. Cell Tissue Res. 2001;306:167–78.
26. Dubreuil RR. Copper cells and stomach acid secretion in the Drosophila midgut. Int J Biochem Cell Biol. 2004;36:745–52.
27. Strand M, Micchelli CA. Quiescent gastric stem cells maintain the adult Drosophila stomach. Proc Natl Acad Sci U S A. 2011;108:17696–701.
28. Strand M, Micchelli CA. Regional control of Drosophila gut stem cell proliferation: EGF establishes GSSC proliferative set point & controls emergence from quiescence. PLoS One. 2013;8:e80608.
29. Oka T, Yamamoto R, Futai M. Multiple genes for vacuolar-type ATPase proteolipids in Caenorhabditis elegans. A new gene, vha-3, has a distinct cell-specific distribution. J Biol Chem. 1998;273:22570–6.
30. Xia D, Zhang Y, Huang X, Sun Y, Zhang H, The C. elegans CBFbeta homolog, BRO-1, regulates the proliferation, differentiation and specification of the stem cell-like seam cell lineages. Dev Biol. 2007;309:259–72.
31. Quante M, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, Lee Y, Friedman R, Asfaha S, Dubeykovskaya Z, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. Cancer Cell. 2012;21:36–51.
32. Quante M, Abrams JA, Lee Y, Wang TC. Barrett esophagus: what a mouse model can teach us about human disease. Cell Cycle. 2012;11:4328–38.
33. Ming SC. Cellular and molecular pathology of gastric carcinoma and precursor lesions: A critical review. Gastric Cancer. 1998;1:31–50.
34. Matkar SS, Durham A, Brice A, Wang TC, Rustgi AK, Hua X. Systemic activation of K-ras rapidly induces gastric hyperplasia and metaplasia in mice. Am J Cancer Res. 2011;1:432–45.
35. Nomura S, Settle SH, Leys CM, Means AL, Peek Jr RM, Leach SD, Wright CV, Coffey RJ, Goldenring JR. Evidence for repatterning of the gastric fundic epithelium associated with Menetrier's disease and TGFalpha overexpression. Gastroenterology. 2005;128:1292–305.
36. Kidd S, Lockett TJ, Young MW. The Notch locus of Drosophila melanogaster. Cell. 1983;34:421–33.
37. Tao J, Chen S, Lee B. Alteration of Notch signaling in skeletal development and disease. Ann N Y Acad Sci. 2010;1192:257–68.
38. Katoh M, Katoh M. Notch signaling in gastrointestinal tract (review). Int J Oncol. 2007;30:247–51.
39. Penon D, Cito L, Giordano A. Novel findings about management of gastric cancer: a summary from 10th IGCC. World J Gastroenterol. 2014;20:8986–92.
40. Carulli AJ, Keeley TM, Demitrack ES, Chung J, Maillard I, Samuelson LC. Notch receptor regulation of intestinal stem cell homeostasis and crypt regeneration. Dev Biol. 2015;402:98–108.
41. Tsai YH, VanDussen KL, Sawey ET, Wade AW, Kasper C, Rakshit S, Bhatt RG, Stoeck A, Maillard I, Crawford HC, et al. ADAM10 regulates Notch function in intestinal stem cells of mice. Gastroenterology. 2014;147:822–34. e813.

42. Du X, Cheng Z, Wang YH, Guo ZH, Zhang SQ, Hu JK, Zhou ZG. Role of Notch signaling pathway in gastric cancer: a meta-analysis of the literature. World J Gastroenterol. 2014;20:9191–9.
43. Wang Z, Da Silva TG, Jin K, Han X, Ranganathan P, Zhu X, Sanchez-Mejias A, Bai F, Li B, Fei DL, et al. Notch signaling drives stemness and tumorigenicity of esophageal adenocarcinoma. Cancer Res. 2014;74:6364–74.
44. Song Y, Li L, Ou Y, Gao Z, Li E, Li X, Zhang W, Wang J, Xu L, Zhou Y, et al. Identification of genomic alterations in oesophageal squamous cell cancer. Nature. 2014;509:91–5.
45. Jia Y, Wang Y, Xie J. The Hedgehog pathway: role in cell differentiation, polarity and proliferation. Arch Toxicol. 2015;89:179–91.
46. Damhofer H, Ebbing EA, Steins A, Welling L, Tol JA, Krishnadath KK, van Leusden T, van de Vijver M, Besselink M, Busch OR, et al. Establishment of patient-derived xenograft models and cell lines for malignancies of the upper gastrointestinal tract. J Transl Med. 2015;13:115.
47. Saqui-Salces M, Merchant JL. Hedgehog signaling and gastrointestinal cancer. Biochim Biophys Acta. 2010;1803:786–95.
48. Kolterud A, Grosse AS, Zacharias WJ, Walton KD, Kretovich KE, Madison BB, Waghray M, Ferris JE, Hu C, Merchant JL, et al. Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. Gastroenterology. 2009;137:618–28.
49. Merchant JL. Hedgehog signalling in gut development, physiology and cancer. J Physiol. 2012;590:421–32.
50. van den Brink GR. Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol Rev. 2007;87:1343–75.
51. Waghray M, Zavros Y, Saqui-Salces M, El-Zaatari M, Alamelumangapuram CB, Todisco A, Eaton KA, Merchant JL. Interleukin-1beta promotes gastric atrophy through suppression of Sonic Hedgehog. Gastroenterology. 2010;138:562–72. 572 e561–562.
52. Dimmler A, Brabletz T, Hlubek F, Hafner M, Rau T, Kirchner T, Faller G. Transcription of sonic hedgehog, a potential factor for gastric morphogenesis and gastric mucosa maintenance, is up-regulated in acidic conditions. Lab Invest. 2003;83:1829–37.
53. Stepan V, Ramamoorthy S, Nitsche H, Zavros Y, Merchant JL, Todisco A. Regulation and function of the sonic hedgehog signal transduction pathway in isolated gastric parietal cells. J Biol Chem. 2005;280:15700–8.
54. Zavros Y, Waghray M, Tessier A, Bai L, Todisco A, Gumucio DL, Samuelson LC, Dlugosz A, Merchant JL. Reduced pepsin a processing of sonic hedgehog in parietal cells precedes gastric atrophy and transformation. J Biol Chem. 2007;282:33265–74.
55. El-Zaatari M, Zavros Y, Tessier A, Waghray M, Lentz S, Gumucio D, Todisco A, Merchant JL. Intracellular calcium release and protein kinase C activation stimulate sonic hedgehog gene expression during gastric acid secretion. Gastroenterology. 2010;139:2061–71. e2062.
56. Schumacher MA, Donnelly JM, Engevik AC, Xiao C, Yang L, Kenny S, Varro A, Hollande F, Samuelson LC, Zavros Y. Gastric Sonic Hedgehog acts as a macrophage chemoattractant during the immune response to Helicobacter pylori. Gastroenterology. 2012;142:1150–9. e1156.
57. Donnelly JM, Chawla A, Houghton J, Zavros Y. Sonic hedgehog mediates the proliferation and recruitment of transformed mesenchymal stem cells to the stomach. PLoS One. 2013;8:e75225.
58. Donnelly JM, Engevik A, Feng R, Xiao C, Boivin GP, Li J, Houghton J, Zavros Y. Mesenchymal stem cells induce epithelial proliferation within the inflamed stomach. Am J Physiol Gastrointest Liver Physiol. 2014;306:G1075–88.
59. Martin J, Donnelly JM, Houghton J, Zavros Y. The role of sonic hedgehog reemergence during gastric cancer. Dig Dis Sci. 2010;55:1516–24.
60. El-Zaatari M, Kao JY, Tessier A, Bai L, Hayes MM, Fontaine C, Eaton KA, Merchant JL. Gli1 deletion prevents helicobacter-induced gastric metaplasia and expansion of myeloid cell subsets. PLoS One. 2013;8:e58935.

61. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature. 2003;425:846–51.
62. Xie K, Abbruzzese JL. Developmental biology informs cancer: the emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers. Cancer Cell. 2003;4:245–7.
63. Fukaya M, Isohata N, Ohta H, Aoyagi K, Ochiya T, Saeki N, Yanagihara K, Nakanishi Y, Taniguchi H, Sakamoto H, et al. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. Gastroenterology. 2006;131:14–29.
64. Hashimoto T, Ogawa R, Matsubara A, Taniguchi H, Sugano K, Ushiama M, Yoshida T, Kanai Y, Sekine S. Familial adenomatous polyposis-associated and sporadic pyloric gland adenomas of the upper gastrointestinal tract share common genetic features. Histopathology. 2015;67(5):689–98.
65. Fox JG, Dangler CA, Whary MT, Edelman W, Kucherlapati R, Wang TC. Mice carrying a truncated *Apc* gene have diminished gastric epithelial proliferation, gastric inflammation, and humoral immunity in reponse to *Helicobacter felis* infection. Cancer Res. 1997;57:3972–8.
66. Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A. 1987;84:5034–7.
67. Davis WJ, Lehmann PZ, Li W. Nuclear PI3K signaling in cell growth and tumorigenesis. Front Cell Dev Biol. 2015;3:24.
68. Shi J, Yao D, Liu W, Wang N, Lv H, Zhang G, Ji M, Xu L, He N, Shi B, et al. Highly frequent PIK3CA amplification is associated with poor prognosis in gastric cancer. BMC Cancer. 2012;12:50.
69. Soutto M, Peng D, Katsha A, Chen Z, Piazuelo MB, Washington MK, Belkhiri A, Correa P, El-Rifai W. Activation of beta-catenin signalling by TFF1 loss promotes cell proliferation and gastric tumorigenesis. Gut. 2014;64(7):1028–39.
70. Simmers MH, Ibsen KH, Berk JE. Concerning the incidence of "spontaneous" stomach cancer in Praomys (Mastomys) natalensis. Cancer Res. 1968;28:1573–6.
71. Nakata H, Matsui T, Ito M, Chiba T. Gastrin/CCK-B receptors on brain, gastric parietal cells and ECL carcinoid tumor of Mastomys natalensis. Kobe J Med Sci. 1993;39:161–70.
72. Martinsen TC, Kawase S, Hakanson R, Torp SH, Fossmark R, Qvigstad G, Sandvik AK, Waldum HL. Spontaneous ECL cell carcinomas in cotton rats: natural course and prevention by a gastrin receptor antagonist. Carcinogenesis. 2003;24:1887–96.
73. Saito T, Inokuchi K, Takayama S, Sugimura T. Sequential morphological changes in N-methyl-N′-nitro-N-nitrosoguanidine carcinogenesis in the glandular stomach of rats. J Natl Cancer Inst. 1970;44:769–83.
74. Abe M, Yamashita S, Kuramoto T, Hirayama Y, Tsukamoto T, Ohta T, Tatematsu M, Ohki M, Takato T, Sugimura T, et al. Global expression analysis of N-methyl-N′-nitro-N-nitrosoguanidine-induced rat stomach carcinomas using oligonucleotide microarrays. Carcinogenesis. 2003;24:861–7.
75. Tatematsu M, Ogawa K, Hoshiya T, Shichino Y, Kato T, Imaida K, Ito N. Induction of adenocarcinomas in the glandular stomach of BALB/c mice treated with N-methyl-N-nitrosourea. Jpn J Cancer Res. 1992;83:915–8.
76. Tatematsu M, Yamamoto M, Iwata H, Fukami H, Yuasa H, Tezuka N, Masui T, Nakanishi H. Induction of glandular stomach cancers in C3H mice treated with N-methyl-N-nitrosourea in the drinking water. Jpn J Cancer Res. 1993;84:1258–64.
77. Yamachika T, Nakanishi H, Inada K, Tsukamoto T, Shimizu N, Kobayashi K, Fukushima S, Tatematsu M. N-methyl-N-nitrosourea concentration-dependent, rather than total intake-dependent, induction of adenocarcinomas in the glandular stomach of BALB/c mice. Jpn J Cancer Res. 1998;89:385–91.
78. Lin SH, Li YH, Leung K, Huang CY, Wang XR. Salt processed food and gastric cancer in a Chinese population. Asian Pac J Cancer Prev. 2014;15:5293–8.

79. Gaddy JA, Radin JN, Loh JT, Zhang F, Washington MK, Peek Jr RM, Algood HM, Cover TL. High dietary salt intake exacerbates Helicobacter pylori-induced gastric carcinogenesis. Infect Immun. 2013;81:2258–67.
80. Furihata C, Ohta H, Katsuyama T. Cause and effect between concentration-dependent tissue damage and temporary cell proliferation in rat stomach mucosa by NaCl, a stomach tumor promoter. Carcinogenesis. 1996;17:401–6.
81. Sorbye H, Svanes C, Stangeland L, Kvinnsland S, Svanes K. Epithelial restitution and cellular proliferation after gastric mucosal damage caused by hypertonic NaCl in rats. Virchows Arch A Pathol Anat Histopathol. 1988;413:445–55.
82. Bergin IL, Sheppard BJ, Fox JG. Helicobacter pylori infection and high dietary salt independently induce atrophic gastritis and intestinal metaplasia in commercially available outbred Mongolian gerbils. Dig Dis Sci. 2003;48:475–85.
83. Fox JG, Dangler CA, Taylor NS, King A, Koh TJ, Wang TC. High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances Helicobacter pylori colonization in C57BL/6 mice. Cancer Res. 1999;59:4823–8.
84. Kodama M, Kodama T, Suzuki H, Kondo K. Effect of rice and salty rice diets on the structure of mouse stomach. Nutr Cancer. 1984;6:135–47.
85. Tatematsu M, Takahashi M, Fukushima S, Hananouchi M, Shirai T. Effects in rats of sodium chloride on experimental gastric cancers induced by N-methyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline-1-oxide. J Natl Cancer Inst. 1975;55:101–6.
86. Takahashi M, Kokubo T, Furukawa F, Kurokawa Y, Tatematsu M, Hayashi Y. Effect of high salt diet on rat gastric carcinogenesis induced by N-methyl-N′-nitro-N-nitrosoguanidine. Gan. 1983;74:28–34.
87. Takahashi M, Nishikawa A, Furukawa F, Enami T, Hasegawa T, Hayashi Y. Dose-dependent promoting effects of sodium chloride (NaCl) on rat glandular stomach carcinogenesis initiated with N-methyl-N′-nitro-N-nitrosoguanidine. Carcinogenesis. 1994;15:1429–32.
88. Toyoda T, Tsukamoto T, Yamamoto M, Ban H, Saito N, Takasu S, Shi L, Saito A, Ito S, Yamamura Y, et al. Gene expression analysis of a Helicobacter pylori-infected and high-salt diet-treated mouse gastric tumor model: identification of CD177 as a novel prognostic factor in patients with gastric cancer. BMC Gastroenterol. 2013;13:122.
89. Kobayashi K, Shimizu N, Tsukamoto T, Inada K, Nakanishi H, Goto K, Mutai M, Tatematsu M. Dose-dependent promoting effects of catechol on glandular stomach carcinogenesis in BALB/c mice initiated with N-methyl-N-nitrosourea. Jpn J Cancer Res. 1997;88:1143–8.
90. Kobayashi K, Inada K, Furihata C, Tsukamoto T, Ikehara Y, Yamamoto M, Tatematsu M. Effects of low dose catechol on glandular stomach carcinogenesis in BALB/c mice initiated with N-methyl-N-nitrosourea. Cancer Lett. 1999;139:167–72.
91. Hirose M, Fukushima S, Tanaka H, Asakawa E, Takahashi S, Ito N. Carcinogenicity of catechol in F344 rats and B6C3F1 mice. Carcinogenesis. 1993;14:525–9.
92. Tanaka H, Hirose M, Hagiwara A, Imaida K, Shirai T, Ito N. Rat strain differences in catechol carcinogenicity to the stomach. Food Chem Toxicol. 1995;33:93–8.
93. Ito N, Fukushima S, Hagiwara A, Shibata M, Ogiso T. Carcinogenicity of butylated hydroxyanisole in F344 rats. J Natl Cancer Inst. 1983;70:343–52.
94. Ito N, Fukushima S, Tsuda H. Carcinogenicity and modification of the carcinogenic response by BHA, BHT, and other antioxidants. Crit Rev Toxicol. 1985;15:109–50.
95. Masui T, Hirose M, Imaida K, Fukushima S, Tamano S, Ito N. Sequential changes of the forestomach of F344 rats, Syrian golden hamsters, and B6C3F1 mice treated with butylated hydroxyanisole. Jpn J Cancer Res. 1986;77:1083–90.
96. Moch RW. Forestomach lesions induced by butylated hydroxyanisole and ethylene dibromide: a scientific and regulatory perspective. Toxicol Pathol. 1988;16:172–83.
97. Ehlers S, Warrelmann M, Hahn H. In search of an animal model for experimental Campylobacter pylori infection: administration of Campylobacter pylori to rodents. Zentralbl Bakteriol Mikrobiol Hyg A. 1988;268:341–6.

98. Cantorna MT, Balish E. Inability of human clinical strains of Helicobacter pylori to colonize the alimentary tract of germfree rodents. Can J Microbiol. 1990;36:237–41.
99. Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain. Gastroenterology. 1997;112:1386–97.
100. Wang X, Willen R, Svensson M, Ljungh A, Wadstrom T. Two-year follow-up of Helicobacter pylori infection in C57BL/6 and Balb/cA mice. APMIS. 2003;111:514–22.
101. Lee A, Fox JG, Otto G, Murphy J. A small animal model of human Helicobacter pylori active chronic gastritis. Gastroenterology. 1990;99:1315–23.
102. Lee A, Chen M, Coltro N, O'Rourke J, Hazell S, Hu P, Li Y. Long term infection of the gastric mucosa with Helicobacter species does induce atrophic gastritis in an animal model of Helicobacter pylori infection. Zentralbl Bakteriol. 1993;280:38–50.
103. Sakagami T, Dixon M, O'Rourke J, Howlett R, Alderuccio F, Vella J, Shimoyama T, Lee A. Atrophic gastric changes in both Helicobacter felis and Helicobacter pylori infected mice are host dependent and separate from antral gastritis. Gut. 1996;39:639–48.
104. Wang TC, Goldenring JR, Dangler C, Ito S, Mueller A, Jeon WK, Koh TJ, Fox JG. Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with Helicobacter felis infection. Gastroenterology. 1998;114:675–89.
105. Fox JG, Sheppard BJ, Dangler CA, Whary MT, Ihrig M, Wang TC. Germ-line p53-targeted disruption inhibits helicobacter-induced premalignant lesions and invasive gastric carcinoma through down- regulation of Th1 proinflammatory responses. Cancer Res. 2002;62:696–702.
106. Mohammadi M, Redline R, Nedrud J, Czinn S. Role of the host in pathogenesis of Helicobacter-associated gastritis: H. felis infection of inbred and congenic mouse strains. Infect Immun. 1996;64:238–45.
107. Thompson LJ, Danon SJ, Wilson JE, O'Rourke JL, Salama NR, Falkow S, Mitchell H, Lee A. Chronic Helicobacter pylori infection with Sydney strain 1 and a newly identified mouse-adapted strain (Sydney strain 2000) in C57BL/6 and BALB/c mice. Infect Immun. 2004;72:4668–79.
108. Kuipers EJ, Nelis GF, Klinkenberg-Knol EC, Snel P, Goldfain D, Kolkman JJ, Festen HP, Dent J, Zeitoun P, Havu N, et al. Cure of Helicobacter pylori infection in patients with reflux oesophagitis treated with long term omeprazole reverses gastritis without exacerbation of reflux disease: results of a randomised controlled trial. Gut. 2004;53:12–20.
109. Mera R, Fontham ET, Bravo LE, Bravo JC, Piazuelo MB, Camargo MC, Correa P. Long term follow up of patients treated for Helicobacter pylori infection. Gut. 2005;54:1536–40.
110. Fuccio L, Zagari RM, Eusebi LH, Laterza L, Cennamo V, Ceroni L, Grilli D, Bazzoli F. Meta-analysis: can Helicobacter pylori eradication treatment reduce the risk for gastric cancer? Ann Intern Med. 2009;151:121–8.
111. Schenk BE, Kuipers EJ, Nelis GF, Bloemena E, Thijs JC, Snel P, Luckers AE, Klinkenberg-Knol EC, Festen HP, Viergever PP, et al. Effect of Helicobacter pylori eradication on chronic gastritis during omeprazole therapy. Gut. 2000;46:615–21.
112. Ley C, Mohar A, Guarner J, Herrera-Goepfert R, Figueroa LS, Halperin D, Johnstone I, Parsonnet J. Helicobacter pylori eradication and gastric preneoplastic conditions: a randomized, double-blind, placebo-controlled trial. Cancer Epidemiol Biomarkers Prev. 2004;13:4–10.
113. Cai X, Carlson J, Stoicov C, Li H, Wang TC, Houghton J. Helicobacter felis eradication restores normal architecture and inhibits gastric cancer progression in C57BL/6 mice. Gastroenterology. 2005;128:1937–52.
114. Lee CW, Rickman B, Rogers AB, Ge Z, Wang TC, Fox JG. Helicobacter pylori eradication prevents progression of gastric cancer in hypergastrinemic INS-GAS mice. Cancer Res. 2008;68:3540–8.
115. Zhang S, Lee DS, Morrissey R, Aponte-Pieras JR, Rogers AB, Moss SF. Early or late antibiotic intervention prevents Helicobacter pylori-induced gastric cancer in a mouse model. Cancer Lett. 2014;355:106–12.

116. Fox JG, Beck P, Dangler CA, Whary MT, Wang TC, Shi HN, Nagler-Anderson C. Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces helicobacter-induced gastric atrophy. Nat Med. 2000;6:536–42.
117. Whary MT, Muthupalani S, Ge Z, Feng Y, Lofgren J, Shi HN, Taylor NS, Correa P, Versalovic J, Wang TC, et al. Helminth co-infection in Helicobacter pylori infected INS-GAS mice attenuates gastric premalignant lesions of epithelial dysplasia and glandular atrophy and preserves colonization resistance of the stomach to lower bowel microbiota. Microbes Infect. 2014;16:345–55.
118. Stoicov C, Whary M, Rogers AB, Lee FS, Klucevsek K, Li H, Cai X, Saffari R, Ge Z, Khan IA, et al. Coinfection modulates inflammatory responses and clinical outcome of Helicobacter felis and Toxoplasma gondii infections. J Immunol. 2004;173:3329–36.
119. Campbell DI, Warren BF, Thomas JE, Figura N, Telford JL, Sullivan PB. The African enigma: low prevalence of gastric atrophy, high prevalence of chronic inflammation in West African adults and children. Helicobacter. 2001;6:263–7.
120. Holcombe C. Helicobacter pylori: the African enigma. Gut. 1992;33:429–31.
121. Takada K. Epstein-Barr virus and gastric carcinoma. Mol Pathol. 2000;53:255–61.
122. van Beek J, Zur Hausen A, Klein Kranenbarg E, van de Velde CJ, Middeldorp JM, van den Brule AJ, Meijer CJ, Bloemena E. EBV-positive gastric adenocarcinomas: a distinct clinicopathologic entity with a low frequency of lymph node involvement. J Clin Oncol. 2004;22:664–70.
123. Lynch HT, Grady W, Suriano G, Huntsman D. Gastric cancer: new genetic developments. J Surg Oncol. 2005;90:114–33. discussion 133.
124. Oh ST, Cha JH, Shin DJ, Yoon SK, Lee SK. Establishment and characterization of an in vivo model for Epstein-Barr virus positive gastric carcinoma. J Med Virol. 2007;79:1343–8.
125. Oh ST, Seo JS, Moon UY, Kang KH, Shin DJ, Yoon SK, Kim WH, Park JG, Lee SK. A naturally derived gastric cancer cell line shows latency I Epstein-Barr virus infection closely resembling EBV-associated gastric cancer. Virology. 2004;320:330–6.
126. Wang TC, Brand SJ. Function and regulation of gastrin in transgenic mice: a review. Yale J Biol Med. 1992;65:705–13.
127. Goldenring JR, Ray GS, Soroka CJ, Smith J, Modlin IM, Meise KS, Coffey Jr RJ. Overexpression of transforming growth factor-alpha alters differentiation of gastric cell lineages. Dig Dis Sci. 1996;41:773–84.
128. Miyazaki Y, Shinomura Y, Tsutsui S, Zushi S, Higashimoto Y, Kanayama S, Higashiyama S, Taniguchi N, Matsuzawa Y. Gastrin induces heparin-binding epidermal growth factor-like growth factor in rat gastric epithelial cells transfected with gastrin receptor. Gastroenterology. 1999;116:78–89.
129. Cui G, Takaishi S, Ai W, Betz KS, Florholmen J, Koh TJ, Houghton J, Pritchard DM, Wang TC. Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. Lab Invest. 2006;86:1037–51.
130. Fox JG, Rogers AB, Ihrig M, Taylor NS, Whary MT, Dockray G, Varro A, Wang TC. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. Cancer Res. 2003;63:942–50.
131. Lofgren JL, Whary MT, Ge Z, Muthupalani S, Taylor NS, Mobley M, Potter A, Varro A, Eibach D, Suerbaum S, et al. Lack of commensal flora in Helicobacter pylori-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. Gastroenterology. 2011;140:210–20.
132. Lertpiriyapong K, Whary MT, Muthupalani S, Lofgren JL, Gamazon ER, Feng Y, Ge Z, Wang TC, Fox JG. Gastric colonisation with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the Helicobacter pylori INS-GAS mouse model of gastric carcinogenesis. Gut. 2014;63:54–63.
133. El-Zaatari M, Tobias A, Grabowska AM, Kumari R, Scotting PJ, Kaye P, Atherton J, Clarke PA, Powe DG, Watson SA. De-regulation of the sonic hedgehog pathway in the InsGas mouse model of gastric carcinogenesis. Br J Cancer. 2007;96:1855–61.

134. Takaishi S, Cui G, Frederick DM, Carlson JE, Houghton J, Varro A, Dockray GJ, Ge Z, Whary MT, Rogers AB, et al. Synergistic inhibitory effects of gastrin and histamine receptor antagonists on helicobacter-induced gastric cancer. Gastroenterology. 2005;128:1965–83.
135. Friis-Hansen L, Sundler F, Li Y, Gillespie PJ, Saunders TL, Greenson JK, Owyang C, Rehfeld JF, Samuelson LC. Impaired gastric acid secretion in gastrin-deficient mice. Am J Physiol. 1998;274:G561–8.
136. Zavros Y, Eaton KA, Kang W, Rathinavelu S, Katukuri V, Kao JY, Samuelson LC, Merchant JL. Chronic gastritis in the hypochlorhydric gastrin-deficient mouse progresses to adenocarcinoma. Oncogene. 2005;24:2354–66.
137. Zavros Y, Rieder G, Ferguson A, Samuelson LC, Merchant JL. Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. Gastroenterology. 2002;122:119–33.
138. Howlett M, Giraud AS, Lescesen H, Jackson CB, Kalantzis A, Van Driel IR, Robb L, Van der Hoek M, Ernst M, Minamoto T, et al. The interleukin-6 family cytokine interleukin-11 regulates homeostatic epithelial cell turnover and promotes gastric tumor development. Gastroenterology. 2009;136:967–77.
139. Kang W, Saqui-Salces M, Zavros Y, Merchant JL. Induction of follistatin precedes gastric transformation in gastrin deficient mice. Biochem Biophys Res Commun. 2008;376:573–7.
140. Saqui-Salces M, Coves-Datson E, Veniaminova NA, Waghray M, Syu LJ, Dlugosz AA, Merchant JL. Inflammation and Gli2 suppress gastrin gene expression in a murine model of antral hyperplasia. PLoS One. 2012;7:e48039.
141. Takaishi S, Tu S, Dubeykovskaya ZA, Whary MT, Muthupalani S, Rickman BH, Rogers AB, Lertkowit N, Varro A, Fox JG, et al. Gastrin is an essential cofactor for helicobacter-associated gastric corpus carcinogenesis in C57BL/6 mice. Am J Pathol. 2009;175:365–75.
142. Beckler AD, Roche JK, Harper JC, Petroni G, Frierson Jr HF, Moskaluk CA, El-Rifai W, Powell SM. Decreased abundance of trefoil factor 1 transcript in the majority of gastric carcinomas. Cancer. 2003;98:2184–91.
143. Fujimoto J, Yasui W, Tahara H, Tahara E, Kudo Y, Yokozaki H. DNA hypermethylation at the pS2 promoter region is associated with early stage of stomach carcinogenesis. Cancer Lett. 2000;149:125–34.
144. Carvalho R, Kayademir T, Soares P, Canedo P, Sousa S, Oliveira C, Leistenschneider P, Seruca R, Gott P, Blin N, et al. Loss of heterozygosity and promoter methylation, but not mutation, may underlie loss of TFF1 in gastric carcinoma. Lab Invest. 2002;82:1319–26.
145. Lefebvre O, Chenard MP, Masson R, Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P, Rio MC. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. Science. 1996;274:259–62.
146. Thiel A, Narko K, Heinonen M, Hemmes A, Tomasetto C, Rio MC, Haglund C, Makela TP, Ristimaki A. Inhibition of cyclooxygenase-2 causes regression of gastric adenomas in trefoil factor 1 deficient mice. Int J Cancer. 2012;131:1032–41.
147. Howlett M, Menheniott TR, Judd LM, Giraud AS. Cytokine signalling via gp130 in gastric cancer. Biochim Biophys Acta. 2009;1793:1623–33.
148. Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, et al. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. Nat Med. 2002;8:1089–97.
149. Judd LM, Alderman BM, Howlett M, Shulkes A, Dow C, Moverley J, Grail D, Jenkins BJ, Ernst M, Giraud AS. Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. Gastroenterology. 2004;126:196–207.
150. Judd LM, Bredin K, Kalantzis A, Jenkins BJ, Ernst M, Giraud AS. STAT3 activation regulates growth, inflammation, and vascularization in a mouse model of gastric tumorigenesis. Gastroenterology. 2006;131:1073–85.
151. Howlett M, Judd LM, Jenkins B, La Gruta NL, Grail D, Ernst M, Giraud AS. Differential regulation of gastric tumor growth by cytokines that signal exclusively through the coreceptor gp130. Gastroenterology. 2005;129:1005–18.

152. Spicer Z, Miller ML, Andringa A, Riddle TM, Duffy JJ, Doetschman T, Shull GE. Stomachs of mice lacking the gastric H, K-ATPase alpha -subunit have achlorhydria, abnormal parietal cells, and ciliated metaplasia. J Biol Chem. 2000;275:21555–65.
153. Judd LM, Andringa A, Rubio CA, Spicer Z, Shull GE, Miller ML. Gastric achlorhydria in H/K-ATPase-deficient (Atp4a(−/−)) mice causes severe hyperplasia, mucocystic metaplasia and upregulation of growth factors. J Gastroenterol Hepatol. 2005;20:1266–78.
154. Lee MP, Ravenel JD, Hu RJ, Lustig LR, Tomaselli G, Berger RD, Brandenburg SA, Litzi TJ, Bunton TE, Limb C, et al. Targeted disruption of the kvlqt1 gene causes deafness and gastric hyperplasia in mice. J Clin Invest. 2000;106:1447–55.
155. Kobayashi T, Tonai S, Ishihara Y, Koga R, Okabe S, Watanabe T. Abnormal functional and morphological regulation of the gastric mucosa in histamine H2 receptor-deficient mice. J Clin Invest. 2000;105:1741–9.
156. Wang WH, Huang JQ, Zheng GF, Lam SK, Karlberg J, Wong BC. Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. J Natl Cancer Inst. 2003;95:1784–91.
157. Dannenberg AJ, Subbaramaiah K. Targeting cyclooxygenase-2 in human neoplasia: rationale and promise. Cancer Cell. 2003;4:431–6.
158. Hahm KB, Song YJ, Oh TY, Lee JS, Surh YJ, Kim YB, Yoo BM, Kim JH, Han SU, Nahm KT, et al. Chemoprevention of Helicobacter pylori-associated gastric carcinogenesis in a mouse model: is it possible? J Biochem Mol Biol. 2003;36:82–94.
159. Leung WK, Wu KC, Wong CY, Cheng AS, Ching AK, Chan AW, Chong WW, Go MY, Yu J, To KF, et al. Transgenic cyclooxygenase-2 expression and high salt enhanced susceptibility to chemical-induced gastric cancer development in mice. Carcinogenesis. 2008;29:1648–54.
160. Hu PJ, Yu J, Zeng ZR, Leung WK, Lin HL, Tang BD, Bai AH, Sung JJ. Chemoprevention of gastric cancer by celecoxib in rats. Gut. 2004;53:195–200.
161. Oshima H, Oshima M, Inaba K, Taketo MM. Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice. EMBO J. 2004;23:1669–78.
162. Oshima M, Oshima H, Matsunaga A, Taketo MM. Hyperplastic gastric tumors with spasmolytic polypeptide-expressing metaplasia caused by tumor necrosis factor-alpha-dependent inflammation in cyclooxygenase-2/microsomal prostaglandin E synthase-1 transgenic mice. Cancer Res. 2005;65:9147–51.
163. Yashiro M, Nishioka N, Hirakawa K. K-ras mutation influences macroscopic features of gastric carcinoma. J Surg Res. 2005;124:74–8.
164. Watari J, Tanaka A, Tanabe H, Sato R, Moriichi K, Zaky A, Okamoto K, Maemoto A, Fujiya M, Ashida T, et al. K-ras mutations and cell kinetics in Helicobacter pylori associated gastric intestinal metaplasia: a comparison before and after eradication in patients with chronic gastritis and gastric cancer. J Clin Pathol. 2007;60:921–6.
165. Brembeck FH, Schreiber FS, Deramaudt TB, Craig L, Rhoades B, Swain G, Grippo P, Stoffers DA, Silberg DG, Rustgi AK. The mutant K-ras oncogene causes pancreatic periductal lymphocytic infiltration and gastric mucous neck cell hyperplasia in transgenic mice. Cancer Res. 2003;63:2005–9.
166. Okumura T, Ericksen RE, Takaishi S, Wang SS, Dubeykovskiy Z, Shibata W, Betz KS, Muthupalani S, Rogers AB, Fox JG, et al. K-ras mutation targeted to gastric tissue progenitor cells results in chronic inflammation, an altered microenvironment, and progression to intraepithelial neoplasia. Cancer Res. 2010;70:8435–45.
167. Ray KC, Bell KM, Yan J, Gu G, Chung CH, Washington MK, Means AL. Epithelial tissues have varying degrees of susceptibility to Kras(G12D)-initiated tumorigenesis in a mouse model. PLoS One. 2011;6:e16786.
168. Kuzushita N, Rogers AB, Monti NA, Whary MT, Park MJ, Aswad BI, Shirin H, Koff A, Eguchi H, Moss SF. p27kip1 deficiency confers susceptibility to gastric carcinogenesis in Helicobacter pylori-infected mice. Gastroenterology. 2005;129:1544–56.

169. Shirin H, Sordillo EM, Kolevska TK, Hibshoosh H, Kawabata Y, Oh SH, Kuebler JF, Delohery T, Weghorst CM, Weinstein IB, et al. Chronic Helicobacter pylori infection induces an apoptosis-resistant phenotype associated with decreased expression of p27(kip1). Infect Immun. 2000;68:5321–8.
170. Wen S, So Y, Singh K, Slingerland JM, Resnick MB, Zhang S, Ruiz V, Moss SF. Promotion of cytoplasmic mislocalization of p27 by Helicobacter pylori in gastric cancer. Oncogene. 2012;31:1771–80.
171. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev. 1996;7:93–102.
172. Yang HK, Kang SH, Kim YS, Won K, Bang YJ, Kim SJ. Truncation of the TGF-beta type II receptor gene results in insensitivity to TGF-beta in human gastric cancer cells. Oncogene. 1999;18:2213–9.
173. Wu MS, Lee CW, Shun CT, Wang HP, Lee WJ, Chang MC, Sheu JC, Lin JT. Distinct clinicopathologic and genetic profiles in sporadic gastric cancer with different mutator phenotypes. Genes Chromosomes Cancer. 2000;27:403–11.
174. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359:693–9.
175. Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, Boivin GP, Bouck N. Thrombospondin-1 is a major activator of TGF-beta1 in vivo [In Process Citation]. Cell. 1998;93:1159–70.
176. Ota M, Horiguchi M, Fang V, Shibahara K, Kadota K, Loomis C, Cammer M, Rifkin DB. Genetic suppression of inflammation blocks the tumor-promoting effects of TGF-beta in gastric tissue. Cancer Res. 2014;74:2642–51.
177. Hahm KB, Lee KM, Kim YB, Hong WS, Lee WH, Han SU, Kim MW, Ahn BO, Oh TY, Lee MH, et al. Conditional loss of TGF-beta signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice. Aliment Pharmacol Ther. 2002;16 Suppl 2:115–27.
178. Nam KT, O'Neal R, Lee YS, Lee YC, Coffey RJ, Goldenring JR. Gastric tumor development in Smad3-deficient mice initiates from forestomach/glandular transition zone along the lesser curvature. Lab Invest. 2012;92:883–95.
179. Takaku K, Miyoshi H, Matsunaga A, Oshima M, Sasaki N, Taketo MM. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res. 1999;59:6113–7.
180. Xu X, Brodie SG, Yang X, Im YH, Parks WT, Chen L, Zhou YX, Weinstein M, Kim SJ, Deng CX. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. Oncogene. 2000;19:1868–74.
181. Kim BG, Li C, Qiao W, Mamura M, Kasprzak B, Anver M, Wolfraim L, Hong S, Mushinski E, Potter M, et al. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Nature. 2006;441:1015–9.
182. Hahn JN, Falck VG, Jirik FR. Smad4 deficiency in T cells leads to the Th17-associated development of premalignant gastroduodenal lesions in mice. J Clin Invest. 2011;121:4030–42.
183. Landgren AM, Landgren O, Gridley G, Dores GM, Linet MS, Morton LM. Autoimmune disease and subsequent risk of developing alimentary tract cancers among 4.5 million US male veterans. Cancer. 2011;117:1163–71.
184. Hemminki K, Liu X, Ji J, Sundquist J, Sundquist K. Autoimmune disease and subsequent digestive tract cancer by histology. Ann Oncol. 2012;23:927–33.
185. Nguyen TL, Khurana SS, Bellone CJ, Capoccia BJ, Sagartz JE, Kesman Jr RA, Mills JC, DiPaolo RJ. Autoimmune gastritis mediated by CD4+ T cells promotes the development of gastric cancer. Cancer Res. 2013;73:2117–26.
186. Nguyen TL, Dipaolo RJ. A new mouse model of inflammation and gastric cancer. Oncoimmunology. 2013;2:e25911.
187. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, et al. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. Nature. 2001;412:99.

188. Figueiredo C, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, Capelinha AF, Quint W, Caldas C, van Doorn LJ, et al. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst. 2002;94:1680–7.
189. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, Betz KS, Penz-Oesterreicher M, Bjorkdahl O, Fox JG, et al. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. Cancer Cell. 2008;14:408–19.
190. Shigematsu Y, Niwa T, Rehnberg E, Toyoda T, Yoshida S, Mori A, Wakabayashi M, Iwakura Y, Ichinose M, Kim YJ, et al. Interleukin-1beta induced by Helicobacter pylori infection enhances mouse gastric carcinogenesis. Cancer Lett. 2013;340:141–7.
191. Goldenring JR, Ray GS, Coffey RJ, Meunier PC, Haley PJ, Barnes TB, Car BD. Reversible drug-induced oxyntic atrophy in rats. Gastroenterology. 2000;118:1080–93.
192. Nomura S, Yamaguchi H, Ogawa M, Wang TC, Lee JR, Goldenring JR. Alterations in gastric mucosal lineages induced by acute oxyntic atrophy in wild-type and gastrin-deficient mice. Am J Physiol Gastrointest Liver Physiol. 2005;288:G362–75.
193. Petersen CP, Weis VG, Nam KT, Sousa JF, Fingleton B, Goldenring JR. Macrophages promote progression of spasmolytic polypeptide-expressing metaplasia after acute loss of parietal cells. Gastroenterology. 2014;146:1727–38. e1728.
194. Li HC, Stoicov C, Rogers AB, Houghton J. Stem cells and cancer: evidence for bone marrow stem cells in epithelial cancers. World J Gastroenterol. 2006;12:363–71.
195. Liu C, Chen Z, Zhang T, Lu Y. Multiple tumor types may originate from bone marrow-derived cells. Neoplasia. 2006;8:716–24.
196. Hutchinson L, Stenstrom B, Chen D, Piperdi B, Levey S, Lyle S, Wang TC, Houghton J. Human Barrett's adenocarcinoma of the esophagus, associated myofibroblasts, and endothelium can arise from bone marrow-derived cells after allogeneic stem cell transplant. Stem Cells Dev. 2011;20:11–7.
197. Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC. Gastric cancer originating from bone marrow-derived cells. Science. 2004;306:1568–71.
198. Yamamoto M, Furihata C, Ogiu T, Tsukamoto T, Inada K, Hirano K, Tatematsu M. Independent variation in susceptibilities of six different mouse strains to induction of pepsinogen-altered pyloric glands and gastric tumor intestinalization by N-methyl-N-nitrosourea. Cancer Lett. 2002;179:121–32.
199. Han SU, Kim YB, Joo HJ, Hahm KB, Lee WH, Cho YK, Kim DY, Kim MW. Helicobacter pylori infection promotes gastric carcinogenesis in a mice model. J Gastroenterol Hepatol. 2002;17:253–61.
200. Tomita H, Takaishi S, Menheniott TR, Yang X, Shibata W, Jin G, Betz KS, Kawakami K, Minamoto T, Tomasetto C, et al. Inhibition of gastric carcinogenesis by the hormone gastrin is mediated by suppression of TFF1 epigenetic silencing. Gastroenterology. 2011;140:879–91.
201. Huh WJ, Khurana SS, Geahlen JH, Kohli K, Waller RA, Mills JC. Tamoxifen induces rapid, reversible atrophy, and metaplasia in mouse stomach. Gastroenterology. 2012;142:21–4. e27.
202. Fox JG, Wang TC, Rogers AB, Poutahidis T, Ge Z, Taylor N, Dangler CA, Israel DA, Krishna U, Gaus K, et al. Host and microbial constituents influence Helicobacter pylori-induced cancer in a murine model of hypergastrinemia. Gastroenterology. 2003;124:1879–90.
203. Ito K, Chuang LS, Ito T, Chang TL, Fukamachi H, Salto-Tellez M, Ito Y. Loss of Runx3 is a key event in inducing precancerous state of the stomach. Gastroenterology. 2011;140:1536–46. e1538.
204. Dempsey PJ, Goldenring JR, Soroka CJ, Modlin IM, McClure RW, Lind CD, Ahlquist DA, Pittelkow MR, Lee DC, Sandgren EP, et al. Possible role of transforming growth factor alpha in the pathogenesis of Ménétrier's disease: supportive evidence from humans and transgenic mice. Gastroenterology. 1992;103:1950–63.

205. Sharp R, Babyatsky MW, Takagi H, Tagerud S, Wang TC, Bockman DE, Brand SJ, Merlino G. Transforming growth factor alpha disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. Development. 1995;121:149–61.
206. Banerjee A, Thamphiwatana S, Carmona EM, Rickman B, Doran KS, Obonyo M. Deficiency of the myeloid differentiation primary response molecule MyD88 leads to an early and rapid development of Helicobacter-induced gastric malignancy. Infect Immun. 2014;82:356–63.
207. Veniaminova NA, Hayes MM, Varney JM, Merchant JL. Conditional deletion of menin results in antral G cell hyperplasia and hypergastrinemia. Am J Physiol Gastrointest Liver Physiol. 2012;303(6):G752–64.
208. Tsuzuki T, Egashira A, Igarashi H, Iwakuma T, Nakatsuru Y, Tominaga Y, Kawate H, Nakao K, Nakamura K, Ide F, et al. Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. Proc Natl Acad Sci U S A. 2001;98:11456–61.
209. Shimada S, Mimata A, Sekine M, Mogushi K, Akiyama Y, Fukamachi H, Jonkers J, Tanaka H, Eishi Y, Yuasa Y. Synergistic tumour suppressor activity of E-cadherin and p53 in a conditional mouse model for metastatic diffuse-type gastric cancer. Gut. 2012;61:344–53.
210. Syder AJ, Karam SM, Mills JC, Ippolito JE, Ansari HR, Farook V, Gordon JI. A transgenic mouse model of metastatic carcinoma involving transdifferentiation of a gastric epithelial lineage progenitor to a neuroendocrine phenotype. Proc Natl Acad Sci U S A. 2004;101:4471–6.

Erratum to: Histopathological, Molecular, and Genetic Profile of Hereditary Diffuse Gastric Cancer: Current Knowledge and Challenges for the Future

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