Current Clinical Pathology
Series Editor: Antonio Giordano

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Gastric Cancer In The Precision Medicine Era

Diagnosis and Therapy

Humana Press

Current Clinical Pathology

Series Editor

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ISSN 2197-781X ISSN 2197-7828 (electronic)
Current Clinical Pathology
ISBN 978-3-030-04860-0 ISBN 978-3-030-04861-7 (eBook)
https://doi.org/10.1007/978-3-030-04861-7

Library of Congress Control Number: 2018962131

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Gastric cancer is an aggressive disease that represents a serious problem and has a daunting impact on global health. Despite an appreciable decrease in incidence over the last several decades, gastric cancer remains one of the most common types of cancer in the world. In recent years, a great progress has been made in understanding the pathogenesis of gastric cancer, especially regarding the importance of *Helicobacter pylori* and its associated inflammatory response. Furthermore, for early and advanced gastric cancers, appropriate treatments have been implemented to maximize curative results, as in the setting of adjuvant oncologic therapies of proven benefit for advanced cases, in addition to surgery.

Our purpose through this book is to provide a general overview of the different aspects of gastric cancer.

The first part aims to clarify the main aspects of tumorigenesis, such as the role of inflammation linked to the presence of *H. pylori* infection, and the genetic and epigenetic mechanisms so far known.

The second part includes the pathological and clinical features and contains information regarding the most recent tissue and serological biomarkers in these neoplasms.

The three successive parts are intended to provide the "state of art" of multimodal treatment approaches to gastric cancer, i.e., standard and novel surgical aspects, common and innovative chemo and radio protocols, and modern targeted therapies. Novel molecular classifications are under consideration to improve diagnostic and prognostic definitions and to prospect future treatments based on the use of immunotherapies and innovative molecules such as noncoding RNA and nanoparticles.

Aviano, Italy — Vincenzo Canzonieri
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Contents

Introduction

About 90–95% of gastric cancers (GC) or stomach cancers are adenocarcinomas. These cancers develop within the cells of the mucosa, the innermost lining of the stomach. Other GC histotypes are lymphoma, gastrointestinal stromal tumors (GISTs), carcinoid tumors, and other rare tumors.

Gastric adenocarcinoma (GAC) is the fourth most common type of cancer and the second most common cause of cancer-related deaths in the world; this is determined, in part, by the late appearance of symptoms, usually associated with disease's advanced stages. In the last decades, the incidence of GAC is declining due to improved nutrition, food preservation, increase in hygiene standards, better prevention, earlier diagnosis and treatment, and *Helicobacter pylori* (*H. pylori*) eradication.

The incidence of GAC varies geographically: more than 50% of new cases of GAC occur in developing countries. The high-risk areas are Eastern Europe, East Asia, and Central and South America; the low-risk areas are North and East Africa, Southern Asia, North America, New Zealand, and Australia [1, 2].

Like other carcinomas, also GAC results from a combination of genetic alterations and environmental factors. Prevention is always the best way to avoid the disease and includes anti-*H. pylori* therapies, healthy diet, chemoprevention, and screening for early cancer detection. Infection with *H. pylori* bacteria seems to be a major cause of stomach cancer, especially cancers in the lower (distal) part of the stomach. Infections caused by this long-lasting germ can cause inflammation (chronic atrophic gastritis) and precancerous alterations of the inner lining of the stomach. An increased risk of stomach cancer is seen in people with diets that have large amounts of smoked foods, salted fish and meat, pickled vegetables, and alcohol drinking abuse. Healthy dietary habits rich in high fresh fruits and vegetables can also lower stomach cancer risk. Furthermore, many studies have confirmed that tobacco smoke increases stomach cancer risk, particularly for cancers of the upper portion of the stomach near the esophagus. Accordingly, the rate of stomach cancer is about doubled in smokers [3, 4].

Only a small percentage of stomach cancers are known to be caused by hereditary diffuse gastric cancer syndrome or by another hereditary cancer syndrome called Lynch syndrome.

From the pathological point of view, GAC mainly consists of two pathological variants, intestinal type and diffuse type. The intestinal type is the end result of an inflammatory process that progresses from chronic gastritis to

atrophic gastritis and finally to intestinal metaplasia and dysplasia. While the intestinal type of gastric cancer is often related to environmental factors such as *Helicobacter pylori* infection, diet, and lifestyle, and it is more common in elderly men, the diffuse type is more often associated with genetic abnormalities [5], and it is more prevalent among women and in individuals under the age of 50. Furthermore, the diffuse type is associated with an unfavorable prognosis because the diagnosis is carried out mainly in advanced stages.

Depending on the site and extent of cancer, surgery is the only potentially curative treatment for all T1b-T4 GACs, and extended lymphadenectomy should be recommended as standard of care in resectable tumors. Endoscopic submucosal resection is the preferred option for early-stage cancer. Furthermore, a survival benefit for postoperative chemotherapy, chemoradiotherapy, and perioperative chemotherapy in case of pathologic $T > 2$ and/or node-positive gastric cancer patients has been established, and chemotherapy should contain 5-fluorouracil and cisplatin or their analogs capecitabine and oxaliplatin. Finally, in select metastatic gastric cancer patients, chemotherapy is better than best supportive care only, with cisplatin-5-fluorouracil or capecitabine as the most widely used drugs. In patients that show HER2 overexpression, the addition of anti-HER2 antibody trastuzumab to first-line chemotherapy is advisable. For HER2-negative patients, two or three combinations, including irinotecan, docetaxel, oxaliplatin, or 5FU prodrugs, are valid treatments. Furthermore, the addition of the anti-VEGFR-2 antibody ramucirumab in second line improves overall survival and progression-free survival when compared to chemotherapy only [6]. The following sections report different aspects related to GC, such as tumorigenesis mechanisms, clinical-pathological features and new molecular classifications, and multimodal treatments ranging from surgical strategies to chemo- and radiotherapy, up to the most recent approaches of precision medicine and the most innovative treatments that involve the use of noncoding RNA, immunotherapy, and nanotechnologies.

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Part I

Gastric Tumorigenesis

Check for updates

1 Gastric Tumorigenesis: Role of Inflammation and *Helicobacter pylori*

Stefania Zanussi, Mariateresa Casarotto, Chiara Pratesi, and Paolo De Paoli

Introduction

Great improvements in molecular and cellular technologies and decades of in-depth studies were needed so that inflammation was added to the hallmarks of cancer, and pioneering observations of Virchow and Coley became widely accepted perspectives to be pursued for translation in cancer cures [1–3]. Inflammation is a coordinated response following infection or tissue damage by exogenous or endogenous agents, which involves innate and adaptive immune system cells and soluble factors. Macrophages, neutrophils, eosinophils, mast cells, dendritic cells (DCs), and natural killer (NK) cells represent the antigen-independent first line of immunological defense against homeostatic perturbation of tissue microenvironment. These cell subsets initiate inflammatory response by sensing pathogen-associated molecular patterns, which are present during microbial infections, and danger-associated molecular patterns, which are components of the host cells released during cell damage or death. At early stages of inflammation, tissue antigens are processed and transported to lymphoid organs by specialized antigen-presenting cells, which allow the activation and expansion of B and T lymphocyte-specific immune responses. In this scenario, intracellular regulatory pathways are activated, which ultimately lead to the secretion of reactive oxygen and reactive nitrogen species (ROS and RNS), of diffusible growth factors, of inflammatory cytokines, and of matrix-remodeling enzymes. These elements induce mobilization and infiltration of additional leucocytes in the affected field and magnify the inflammatory reaction until the resolution of the injury or infection.

The tumorigenic fate of the immune response largely depends on the physiological state of the epithelial, stromal, and vascular microenvironment and on the immune cell profile that are part of it, hence from the signals conveyed toward autophagy/death, differentiation, proliferation, and angiogenetic circuits and from the cross talk between them. The duration of the inflammation is another key feature affecting the outcome of the immune responses. This is strictly linked to the presence of host immunogenetic predisposition and/or ongoing chemical, physical, or biological irritation. In the case of gastric mucosa, infection with persistent microorganisms bearing oncogenic potential such as Epstein-Barr virus

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_1

and *Helicobacter pylori* (*H. pylori*) can initiate local inflammation and, after elusion of immune clearance mechanisms, may cause chronic inflammation. Specific or non-specific viral and bacterial virulence factors in conjunction with immunity defects can cause aberrant interactions between microbes and gastric epithelial cells. This condition can drive premalignancy, implementing the inflammatory response with the accumulation of new genetic and epigenetic modifications in epithelial cells, actually favoring the establishment of a gastric cancerized field.

Helicobacter and Inflammation: The Two Facet Janus

H. pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium colonizing the human stomach. From a biological and evolutionary point of view, *H. pylori* has coevolved with humans for at least 50,000 years to be transmitted from person to person and become a commensal of the stomach [4, 5]. An homeostatic equilibrium between bacterial effectors and host responses allows microbial persistence, but also confers the risk of gastric neoplasia. In 1994 *H. pylori* was classified as a class I human carcinogen by the International Agency for Research on Cancer working group for its association with an increased risk for gastric cancer, in particular non-cardia gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma [6]. Since that time, *H. pylori* infection is considered the primary cause of gastric neoplasms [7], although the etiology is multifactorial. One of the first mechanisms by which *H. pylori* may express its pathogenetic potential is inflammation-related and refers to the production of autoreactive immunoglobulins; these may cause complement-dependent cell lysis and small immune complexes formation that may promote local damage [8]. Autoantibodies originate through molecular mimicry of host epitopes by lipopolysaccharide (LPS) structures of *H. pylori* [9]. These observations prompt to evaluate the inflammation-related carcinogenic potential of the structural components of a broad range of microbial populations colonizing the gastric environment.

Both the undifferentiated and the differentiated gastric cancer types (named diffuse-type carcinoma and intestinal-type adenocarcinoma, respectively) are associated with *H. pylori*. However, only the pathogenesis of the intestinal cancer seems to significantly involve the chronic inflammation, which directs the abnormal differentiation of the normal gastric mucosa toward the precancerous gastric lesions. This can be done according to the cascade model hypothesized by Correa, which involves the evolution of the forms of non-atrophic gastritis, toward multifocal atrophic gastritis without intestinal metaplasia, intestinal metaplasia, dysplasia, and finally cancer [10]. All these lesions occur in a setting of inflammation and in a complex milieu of diffusible factors. Despite the variable, but significant, prevalence of *H. pylori* infection in various countries [11], it is estimated that 1–3% of infected people will develop non-cardia gastric cancer and lymphoma [12, 13]. Indeed, besides the environmental factors, such as smoking and diet, and the commensal microbes, the clinical outcome of the infection is conditioned by virulence factors of *H. pylori*, by its high phenotypic and genomic heterogeneity within the gastric niche [14] as well as by genetic susceptibility and immune profile of the host [15].

Each host is not colonized by a single type of *H. pylori,* but by a multitude of genetically closely related microorganisms similar to quasispecies, which interfere with signaling pathways influencing host cell growth and death [16, 17]. From an ecological and teleological point of view, the diversity is originated by the bacterium in an attempt to persist in the microenvironment, notwithstanding the oxidative stress directly caused by *H. pylori* virulence factors and indirectly by inflammatory response. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)1-β and IL-8, sustain inflammation in gastric mucosa, but anti-inflammatory cytokines, such as IL-10, tend to turn it off. They are released by several components of the immune system as well as by cells immersed in the stromal microenvironment, such as fibroblasts, epithelial, and endothelial cells. They can accomplish pleiotropic effects on a wide range of cell types, including immune and epithelial cells. Since variations in genotypes heightening cytokine levels have

been associated with an increased risk of gastric cancer [18–22], cytokines are believed to enhance overall rather than attenuate the pathogenicity of the bacterium. However, it's elemental to highlight that the cellular composition of the microenvironment might deeply influence the cancer risk; the own different $CD4^+$ T cell subsets can secrete different cytokine and chemokine types, which in turn can stimulate different signal transduction pathways and activation of transcription factors, leading to pro-inflammatory reactive or anti-inflammatory suppressive responses.

Helicobacter pylori-Specific Determinants Affecting Inflammation and Tumorigenesis

A plenty of virulence factors have been described in *H. pylori* infection. Some of them are highly studied and specifically involved in inflammatory response after infection. Moreover, they cooperate to the inflammation-related tumorigenic process. Among the most mentioned virulence determinants for their relevance in colonization, persistence, and oxidative stress induction, there are the *H. pylori* neutrophil-activating protein (HP-NAP), the γ-glutamyl-transpeptidase (GGT), the cytotoxin-associated gene pathogenicity island (*Cag*PAI), and the vacuolating cytotoxin A (VacA).

While GGT and HP-NAP are constitutively expressed and show little genetic variability among *H. pylori* isolates, perhaps indicating a structural function or a lack of immune selection for diversification [23], on the other hand, *vacA* and *Cag*PAI show plasticity, being apt to genetic modifications which modulate their virulence [24–26]. The characteristics and modalities of action of these different virulence factors are summarized in the following paragraphs.

H. pylori Neutrophil-Activating Protein

H. pylori neutrophil-activating protein (HP-NAP) has probably evolved as a pro-inflammatory molecule to sustain the production of reactive oxygen intermediates by human neutrophils, functional to the release of nutrients, which can speed *H. pylori* growth [27]. It has been described that HP-NAP can trigger inflammation in conjunction with other bacterial and host-derived factors [28], but also as an only molecule. Indeed, several studies sustain a model in which HP-NAP represents a critical element in initiating the inflammatory process. HP-NAP is probably released after cell lysis in the infected mucosa of the stomach, and, after its transfer through gastric epithelial lining, it activates subepithelial resident mast cells and macrophages [29]. Consequently, these innate immune components release biochemical mediators and, in particular, the pleiotropic cytokine TNF-α. Overall, soluble factors attract and stimulate the adhesion and extravasation of polymorphonucleates (PMN) and lympho-monocytes through the endothelium lining the vessels, as suggested by the TNF-α-induced upregulation of adhesion molecules V-CAM and I-CAM on the surface of endothelial cells and by *in vitro* and *in vivo* experiments on animal models [30, 31]. PMN and monocytes produce and secrete ROS through the HP-NAP-induced increase of cytoplasmic Ca^{2+} and phosphorylation of proteins, leading to assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on plasma membrane. Moreover, PMN and monocytes are activated to secrete cytokines and chemokines which amplify the inflammatory state. Among these, IL-12 and IL-23 contribute to differentiate the monocytes into a mature dendritic phenotype and the T- lymphocytic response toward a cytotoxic T- helper type 1 (Th1) phenotype producing interferon-γ (IFN-γ), TNF-β, IL-12, IL-18, IL-17, and TNF-α [32]. Preclinical studies demonstrate that HP-NAP inhibits the differentiation of Th0 into Th2 profile [23].

γ-Glutamyl-Transpeptidase

γ-Glutamyl-transpeptidase (GGT) is a virulence factor virtually associated with all wild-type *H. pylori* strains, although strain-to-strain variations in GGT expression among clinical isolates from patients with different disease statuses have been observed. GGT is related to ROS production

from the epithelium and to oxidation of DNA and membrane lipids by using the body's master antioxidant glutathione (GSH), which is catabolized by GGT itself. Besides pro-apoptotic and necrotic effects evoked by ROS compounds and potentially sustained by other virulence factors such as VacA, GGT shows anti-apoptotic activities by activation of p38 mitogen-activated protein kinases (MAPKs), protein kinase B (AKT), and nuclear factor k-light-chain-enhancer of activated B cell (NF-kB) signaling pathways; the subsequent production of inducible nitric oxide synthase (iNOS), DNA damage, IL-8, and prostaglandin synthase cyclooxygenase-2 (COX-2) enhances the inflammatory reaction and induces epithelial cell proliferation [33]. Additionally, GGT suppresses T cell proliferation by inducing cell cycle arrest through the disruption of the Ras signaling pathway [34]. *In vitro* and *in vivo* studies suggest that GGT contributes to DC tolerization and directs the T cell response toward a regulatory immunosuppressive phenotype [35]. A suppressive milieu inhibits lymphocyte activation and favors *H. pylori* escape and persistent infection.

Cytotoxin-Associated Gene A Pathogenicity Island and Vacuolating Cytotoxin A

Cytotoxin-associated gene A pathogenicity island (*Cag*PAI) is a 40,000 base pairs sequence containing coding regions for virulence determinants and several proteins participating to the assembly of a specialized syringe machinery called type IV secretion system. Through this structure, *H. pylori* is able to inject into cells inflammation- and tumorigenesis-related bacterial components, such as the cytotoxin-associated gene A (CagA), peptidoglycans, and methyltransferases. Proteins encoded by *Cag*PAI genes induce inflammation by using the host signaling pathways essential for maintenance of the normal gastric mucosa homeostasis [36]. In the case of CagA, after translocation into epithelial cells, it acts through direct interaction with intracellular receptors in a phosphorylation-dependent or phosphorylation-independent manner. In the first case, CagA becomes phosphorylated by members of the Src and Abl family kinases at specific amino acidic motifs in the C-terminus of the protein (Glu-Pro-Ile-Tyr-Ala, EPIYA). This phosphorylation allows CagA binding to SH2 domain-containing proteins, such as SHP2 tyrosine phosphatase, causing its activation and subsequent induction of the extracellular signal-regulated kinases (ERK)-MAPK pathway, which leads to mitogenic response and cellular migration [37]. In the second case, CagA is translocated, but not phosphorylated, and it determines altered activation of β-catenin, disruption of apical junctional complexes, and loss of cellular polarity. Moreover, non-phosphorylated CagA targets a series of adhesion, enzymatic, and transducer molecules, which leads to mitogenic and pro-inflammatory responses [38–41]. CagA also interacts with tumor suppressor proteins, such as Runt-related transcription factor 3 (RUNX3) and protein 53 (p53) leading to their proteasomal degradation [37]. It has been reported that translocated CagA into the host cell is degraded by oxidative stress-dependent autophagy and, hence, short-lived, except when it enters $CD44v9^{+}$ gastric cancer stem-like cells, that show oxidative stress resistance due to their high GSH content [42]. The expression of the CD44 homing receptor can be induced upon chronic inflammation [43], is involved in the upregulation of GSH synthesis, contributes to the progression of precancerous gastric lesions in patients with *H. pylori* infection, and correlates positively with recurrence of gastric cancer [44–46]. These observations suggest that the accumulation of alterations due to ROS and the cell survival through protection against ROS may play a considerable role for the generation of cancer cells in the infected gastric mucosa.

CagPAI-codified type IV secretion system can also deliver peptidoglycans into host cells, where they are recognized by the nucleotide-binding oligomerization domain-containing protein 1 (NOD1). The subsequent activation of NF-kB, p38, and extracellular ERK signaling induces the production of pro-inflammatory cytokines macrophage inflammatory protein (MIP)-2, β-defensins,

and IL-8. Additionally, the interaction between NOD1 and post-translational-modified peptidoglycans modulates the production of type I interferons which are involved in the activation of DCs and of T cell cytotoxic effector functions [47–49].

CagA and other *H. pylori* molecules can be injected not only into gastric epithelial cells, but also into B lymphoid cells and DCs. As a consequence, host's immune responses can be suppressed through the reduction in the secretion of pro-inflammatory cytokines, such as IL-12p40, and the increase in the expression of suppressive cytokines, such as IL-10 [50]. This highlights the existence of pro-inflammatory and anti-inflammatory effects produced by the same virulence component in dependence on the cellular metabolic status and composition of the microenvironment.

VacA is a pore-forming protein which is secreted by *H. pylori* through a type V autotransport secretion system. It exerts multiple effects on epithelial and immune cells in synergy with other virulence determinants. VacA can be internalized into the host cells by endocytosis; afterward it accumulates in different cellular compartments inducing apoptosis. In parallel, it contributes to the successful colonization of the gastric niche disrupting epithelial cell tight connections and allowing the access of bacterial molecules and *H. pylori* to the lamina propria. This function is shared with CagA that is able to bind and inhibit PAR1b, a protein essential for the establishment and maintenance of cell polarity. Once in the innermost layers of the gastric mucosa, VacA encounters granulocytes and T cells recruited to the sites of infection by the triggered inflammation program. Herein, VacA is capable of inducing an influx of Ca^{2+}, probably NF-kB activation, and consequent inflammation through generation of oxidative stress and IL-8 secretion [51, 52]. On the other hand, it modulates the inflammatory response restricting T lymphocytes proliferation and effector functions [53]. *In vitro* and *in vivo* experiments demonstrate that VacA, in cooperation with GGT, contributes critically and non-redundantly to *H. pylori* tolerizing effects on murine DCs allowing persistence of the bacterium [35, 54].

Helicobacter pylori Affects Early Phases of Inflammation

Several evidences point to an involvement of *H. pylori* in the first phases of the carcinogenesis while long lasting molecular changes in epithelial cells, which result from the initial infection with virulent *H. pylori* strains, contribute to tissue damage progression [55, 56]. Indeed, the reversibility of oxidative and nitrosative stress processes, one of the crucial initial steps of the inflammatory reaction contributing to carcinogenesis in gastric mucosa, has been documented after *H. pylori* eradication [57]. Moreover, prospective studies show that *H. pylori* eradication by antibiotics reduces the incidence of precancerous lesions, and it is effective in reversing atrophic gastritis, but not intestinal metaplasia [58, 59]. Finally, *H. pylori* eradication does not decrease the risk of gastric cancer in patients with more advanced metaplastic or dysplastic mucosal lesions [60].

Inflammation and *H. pylori*-Mediated Oxidative and Nitrosative Stresses

RNS are produced mainly by neutrophils and macrophages, but also by gastric epithelial cells through the action of the nitric oxide synthase (NOS) and, especially, of iNOS. Nitric oxide (NO) is sufficiently long-lived to diffuse through the extracellular matrix and enter the nucleus of epithelial cells infected by *H. pylori* and those surrounding them within the gastric pit. ROS, such as superoxide (O_2^-), is active in this biochemical pathway. The source of effective ROS is the epithelial cell itself, since ROS generated by neutrophils and macrophages are not sufficiently long-lived to diffuse through extracellular matrix and penetrate epithelial cell membranes. Here, NO and O_2^- react to form peroxynitrite ($ONOO^-$), which causes DNA damage through guanine nitration and, finally, mutations, impairment of DNA repair enzymes and genomic instability [61–63]. Changes in lipid and protein expression consequent to oxidative stress have been observed [64, 65]. Last but not least, induced NO production interferes with transcriptional modulation by

promoting DNA hyper-methylation both in non-coding and coding sequences for clincher proteins of the carcinogenetic intracellular pathways, such as p53, the cyclin-dependent kinase inhibitors (CDKN2A/CDKN2B), the epithelial cadherin-1 (CDH1) or mutL homolog 1 (MLH1), and many others. It's worth noting that passenger genes, namely, genes that are not directly causally involved in gastric carcinogenesis, are even significantly subjected to silencing by aberrant methylation in the cancerized field [55, 66, 67]. These observations point to methylation rather than silencing of genes by mutation as the main mechanism for inactivation of driver and passenger tumor suppressor genes, indicating that gastric cancer is an epigenetic disease [56].

ROS accumulation in differentiated and stem gastric cells can be directly and indirectly induced by *H. pylori*. Due to its poor immunogenicity, LPS helps the bacteria to develop a chronic infection and, following activation of epithelial Toll-like receptor (TLR) 4 signaling, contributes to epithelial cell ROS production [68, 69]. Moreover, especially highly virulent CagA$^+$ *H. pylori* strains can cause pro-oxidant activities through induction of NADPH oxidase or spermidine oxidase activity in host gastric cells [70–72]. ROS generation is indirectly induced by *H. pylori* infection through interaction of TNF-α-receptor on mucosal cell surface with TNF-α released by inflammatory cells in response to the infection. Epigenetic modifications can be directly induced by *H. pylori* possessing a functional type IV secretion system [36]. Indeed, through this structure, specific methyltransferases encoded by *H. pylori* may be injected into the host cell [73]. However, studies in gerbil-based models of carcinogenesis evidenced a major role of *H. pylori*-induced inflammation rather than a unique direct role of *H. pylori*-specific virulence factors in DNA methylation modulation. Indeed, increases of iNOS, IL1-β, TNF-α, and CXCL2 transcription, which are consequent to and synergistic with *H. pylori* infection immunopathologic effects, were shown to parallel the DNA methylation levels in gastric mucosa [74, 75]. Further experiments in animal models suggest that infiltrating mucosa monocytes are central components for *H. pylori*-dependent methylation induction and that the specificity of aberrant gene methylation in target cells is conditioned from their genomic architecture and from epigenetic elements already present in the cells where methylation is activated [56].

Inflammation and *H. pylori*-Mediated Alteration of DNA Repair Mechanisms

H. pylori may affect activation-induced cytidine deaminase (AID), which is an inducible enzyme, physiologically responsible for editing the human genome, e.g., for generating genomic diversity within the variable regions of immunoglobulin genes in activated B lymphocytes through somatic hypermutation and class switch recombination. AID is not expressed in normal gastric mucosa, but it is overexpressed in a proportion of *H. pylori*-infected gastric epithelium and in gastric cancer tissues, especially in the presence of mononuclear cell infiltration and intestinal metaplasia [76]. Most importantly, AID expression decreases after *H. pylori* eradication, suggesting a cause-effect link with the bacterium [77]. *Cag*PAI$^+$, but not *cag*PAI$^-$ *H. pylori* isolates, are able to stimulate aberrant AID expression in epithelial cell lines, causing chromosomal aberrations and somatic point mutations in tumor suppressor genes such as the aforementioned *p53* and *CDKN2A/CDKN2B* [76, 78]. Moreover, also pro-inflammatory cytokines, such as TNF-α, indirectly increase the expression of AID through NF-kB intracellular pathway activation [55].

In vitro experiments on infected gastric epithelial cells demonstrate *H. pylori*-induced downregulation of proteins that are sequentially involved in the mechanism of base excision repair (BER) and which mediate the removal of incorrect single base residues [79]. Also the proteins of the DNA base mismatch repair are downregulated by *H. pylori* infection in gastric epithelial cells as well as in a *H. pylori*-infected mouse model and in *H. pylori*-positive patients with chronic gastritis [80, 81].

In gastric cell lines and primary gastric epithelial cells, it has been demonstrated that *H. pylori* infection prompts downregulation of several components engaged in double-strand DNA break (DSB) repair pathway, which generate carcinoge-

netic lesions if they are not appropriately restored [61]. Indeed, DNA damage affecting chromosomal ends resulting in telomere shortening and chromosomal instability has been reported [61]. Even if the precise mechanisms by which these events occur are not completely understood and elucidated, *ex vivo* and *in vitro* studies demonstrate that *H. pylori* infection is associated with alteration in DNA repair by direct host-pathogen contact, and prolonged infection may result in unrepaired breaks [82]. A genome-wide screening in gastric epithelial cell lines suggests an involvement of a type IV secretion system-dependent injection of XPF/XPG endonucleases together with NF-kB activation in DSB induction [83].

Toward Cellular Autophagy or Death

The molecular damage in gastric epithelial and immune cells, consequent to the activation of oxidative stress pathways by *H. pylori*-induced inflammation, stimulates caspase-mediated autophagy or apoptosis, with a raise in cell turnover during the initial steps of the infection [84, 85]. Autophagy is an intrinsic cytoprotective mechanism by self-eating and recycling of cellular components. Hence, autophagy can suppress tumor initiation by preserving normal cells and inhibiting inflammation. However, it can promote proliferation of damaged cells with precancerous characteristics by favoring inflammatory cell growth and providing sufficient oxygen and nutrients [86]. Hence, an increase in cell survival and proliferation may be induced in infected and in neighboring cells, adding on the possibility of malignant characteristics acquirement thanks to accumulation of mutagenic DNA lesions, altered methylation, and block of the DNA repair machinery [87]. VacA is important in autophagy induction through the formation of autophagosome, a double membrane structure encapsulating intracellular and pathogen-derived damaged organelles and proteins, among them VacA itself and CagA, whose activities are modulated [84, 88]. As the chronic infection establishes and progresses, DNA damage may determine aberrations in autophagy-associated proteins, such as the oncoprotein p62/SQSTM1, which is overexpressed in gastric lesions and has been found to promote tumorigenesis through the NF-kB signaling transduction pathway [89, 90].

In vitro studies demonstrate that autophagy and apoptosis are molecular mechanisms which could cross talk between them and may control the cell fate in autonomous or cooperative ways [91, 92]. The selected pathway seems to be dependent on the cellular surface receptor status, such as the presence of TRAIL or CD95, on Bcl-2 as a central regulator of autophagy and apoptosis, and on the intracellular signaling milieu [93–95]. A key intracellular component driving death and autophagy is the inflammasome, a cytosolic multiprotein oligomer containing caspases, whose exact composition depends on the activator which initiates inflammasome assembly. Inflammasome has dual opposite roles in the oncogenesis: one in the anti-tumor inflammatory response by eliminating precancerous precursors through apoptosis and, on the other hand, a pro-tumorigenic effect by stimulating production of trophic factors for precancerous cells and stroma [96]. Anti-tumorigenic and pro-tumorigenic properties are largely determined by the types of cells, tissues, and organs involved [97]. For instance, some cells with DNA damage elicited by CagA$^+$ *H. pylori* strains are less likely to undergo apoptosis, and thus they are at high risk of malignant transformation [72]. This highlights that the interplay between the host and different *H. pylori* strains with differentially expressed virulence determinants is complex and may strongly influence the progression of the disease.

The Progression of *H. pylori*-Induced Precancerous Lesions: A Continuous Tolerizing Relationship

Beyond the biochemical, genetic, and molecular mechanisms triggered in the early phases of *H. pylori* infection and of inflammation, cellular and soluble factors deeply influence the relationships between *H. pylori* and the gastric microenvironment, sustaining bacterial persistence and sur-

vival of those cells altered from inflammation and that are shaped toward a precancerous lesion. Pro-inflammatory factors derived from damaged cells, such as IL-1β, and from activated T lymphocytes, such as IFN-γ, IL-4, IL-10, and IL-12, trigger immunosuppressive pathways from myeloid cells [98]. In addition, $CD4^+$ T cells recruited in the inflamed microenvironment secrete pleiotropic chemokines and cytokines, which play a fundamental role in the final clinical outcome of the infection and of the cancerized field, through the activation of many pathways, such as those leading to epithelial-to-mesenchymal transition or development of gastric cancer stem cells [99–103].

Mechanisms of *H. pylori* Attenuation and Evasion from Immune Surveillance

Despite the activation of a strong immune response, *H. pylori* is able to sustain the infection for several years or throughout lifetime. *H. pylori* survives oxidative stress by the production of enzyme oxidase and superoxide catalase, thus determining its persistence in the gastric mucosa and the further enhancement of the oxidative burst. Strains with high virulence levels and carriers of bacterial determinants with toxic activity seem to account for a high risk of gastric cancer development [26, 104]. However, the *H. pylori* gastric niche harbors bacterial strains with differential virulence acquired by genetic recombination as a strategy for survival and persistence in the site of infection. Indeed, DNA damage induced by inflammation in epithelial and stromal cells can involve not only the host genetic background, but also the *H. pylori* genome. Homologous recombination can act as a repair pathway of DNA breaks, prompting antigenic variation in *H. pylori* [105]; for instance, rearrangements in the genes encoding post-translational modifying enzymes, such as alpha-fucosyltransferases or peptidoglycan deacetylases, can determine changes in their activity with modulation of bacterial cell wall antigenic specificity [106, 107].

Molecular biology studies suggest functional relationships between different genomic traits of *H. pylori*. In particular, the composition of the *Cag*PAI greatly affects bacterial motility, survival capacity in different gastric microenvironments, production of pro-inflammatory cytokines, and antimicrobial susceptibility [108–112]. It has been highlighted that a single infective *H. pylori* strain may include variable proportions of subtypes with different *Cag*PAI genotypes, a phenomenon consistent with host-induced adaptive changes of the bacterial population infecting the stomach [113, 114]. Indeed, heterogeneous genomic and proteomic profiles of *H. pylori* strains and subtypes have been described showing a tendency to an association with different precancerous or pathologic conditions [26, 115–117]. Deletions of *Cag*PAI genes are more frequently detected among individuals with metaplasia and atrophic gastritis than non-atrophic gastritis or duodenal ulcers [118, 119]. These mechanisms entail virulence attenuation favoring colonization and persistence, but also modify the interaction capacity of the bacterium favoring the escape from the immunosurveillance.

Myeloid and Lymphoid Cellular and Soluble Factors Affecting the Clinical Outcome of *H. pylori* Infection

The secretion of inflammatory cytokines from healthy and damaged cells can be promoted by interaction of bacterial LPS, flagellins, toxins, and cellular products with membrane receptors, such as TLRs, or cytosolic components, such as inflammasomes. A paradigmatic example of membrane receptor is TLR4. It is expressed in immune as well as epithelial and stromal cells, where it can activate MyD88-dependent pathways, with the transcription of genes encoding for pro-inflammatory cytokines and chemokines (TNF, IL-1β, IL-18), immunosuppressive cytokines (IL-10 and transforming growth factor (TGF)-β), and angiogenic mediators (vascular endothelial growth factor (VEGF), epidermal growth factor (EGF)). In addition, TLR4 has

been detected in tumoral cells, where it is capable of activating mitogen-activated protein kinases (MAPK) and NF-kB, suggesting its direct role in apoptosis inhibition and proliferation stimulation [120]. Inflammasomes are predominantly expressed in macrophages and can promote cytokine and chemokine production as well, especially IL-18 and IL-1β. The *Cag*PAI-encoded type IV secretion system, LPS, VacA, and bacterial urease B subunit seem to play a role in inflammasome activation. Recent studies highlight that the *H. pylori*-induced inflammasome activation and consequent IL-18 and IL-1β secretion need the coordinated cooperation between TLR-2, Nod-like receptor family pyrin domain-containing 3 (NLRP3) and caspase-1 [121].

IL-18 is a multifactorial chemokine, which intervenes directly activating $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ naïve T cells that acquire a Th1-IFN-γ-secreting phenotype under the synergic action of IL-12 [99, 122]. Besides this anti-inflammatory action, mucosa integrity protection, and anti-cancer effect, IL-18 manifests pro-cancer properties [123]. This effect seems to be related to an impaired NK cell function through a PD-1-dependent mechanism, as it has been evidenced by *in vitro* and murine models [124]. However, IL-18 role in gastric cancer is not clearly understood in the clinical settings [125]. Overexpression of IL-1β is involved in the pathogenesis of gastric cancer through an immune-tolerizing effect of the mucosal gastric microenvironment guided by the mobilization of myeloid-derived suppressor cells (MDSCs) to the stomach [126]. MDSCs are one of the representative immune suppressive cells having the capacity to increase T cell apoptosis and suppress T cell responses, directing the result of the infection toward evasion from immune system and pathology [127]. MDSC levels are significantly increased in cancer patients and correlate with cancer clinical stages and poor prognosis [128–130], to such an extent that they have been mentioned as possible prognostic biomarkers of gastric cancer together with macrophages, neutrophils, and DCs [131, 132]. Finally, IL-1β production by MDSCs may induce secretion of IL-17 by CD4+ T cells [102].

Th17

Th17 and Th1 are the predominant subsets during the inflammatory phases of *H. pylori* infection, with Th17 response involved at earlier stages of infection than Th1 response [133]. In particular, $CagA^+$ strains stimulate DCs to IL-1β and IL-23 production. In the presence of antigen presentation, DCs activate $CD4^+$ naïve T cells to differentiation toward a Th17 phenotype. At intracellular level, the process is controlled by signal transducers, such as Signal Transducer and Activator of Transcription-3 (STAT-3), and by the transcription factors retinoic acid receptor-Related Orphan Receptors (ROR) γt and α. TGF-β, BAFF, and IL-6 secreted by DCs may be additional important factors for Th17 differentiation. They act through STAT-3 and NF-kB pathways [134]. In particular, TGF-β induces the expression of both RORγt and of forkhead box P3 (FoxP3) in naïve T cells; the latter molecule is a transcription factor capable of suppressing the activation of RORγt by a physical interaction and of deviating the differentiation of naïve T cells toward an immunosuppressive T regulatory (Treg) signature [135]. IL-6 links the differentiative pathways of Th17 and Treg, by activating STAT-3 pathway and down-modulating FoxP3 expression, finally unbalancing the ratio between these two subsets in favor of Th17. IL-6 expression is high in *H. pylori*-infected subjects as well as in physiological aging, where this cytokine is involved in the maintenance of a low level of systemic and local chronic inflammation, that can unbalance immune system functions toward tolerance and senescence, with a high risk of morbidity [136, 137].

Th17 cell subsets are able to release several chemokines and cytokines, namely, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-26, TNF-α, CCL20, and GM-CSF, although not all are Th17 specific. Epithelial cells and fibroblasts are stimulated by Th17 cytokines/chemokines toward pro-inflammatory soluble factors secretion, further recalling infiltration of macrophages, activated monocytes, T cells, and DCs in the microenvironment. Functional for tissue remodeling, but of relevance for re-localization of cells with malignant or premalignant characteristics,

Th17 cells stimulate epithelial cells to produce matrix metalloproteinases (MMP) that disrupt microenvironment architecture. Although IL-17 responses are downregulated by immunosuppressive enzymes, such as indoleamine 2,3-dioxygenase (IDO), or by reduced expression of co-stimulatory receptors on the Th17 surface, the activity of this T cell subset continues also after disappearance of the bacterium thanks to the action of IL-1β, which levels remain elevated in the gastric mucosa [138].

Th1

Th1 cells are involved primarily in defense against intracellular pathogens and in the isotypic switch of immunoglobulins to isotypes with complement-activation properties. *H. pylori* colonization of gastric mucosa seems to be directly proportional to Th1 immune response, since an insufficiency in this lineage is associated with enhanced bacterial density [139]. Outer membrane proteins of *H. pylori* induce NK and DC activation and maturation with predominant production of IL-12, IL-18, and IFN-γ. The synergic action of DCs and NK cells and their soluble mediators induces the expression of the transcription factor T-box expressed in T cells (T-bet) in T cell receptor (TCR)-engaged naïve $CD4^+$ T cells, leading to their differentiation in Th1 secreting at least IL-2 and IFN-γ. Hence, through an autocrine mechanism, IFN-γ enforces the Th1 polarization operated by NK, while IL-2 stimulates the progression of target cells from G0 to G1 phase, initiating the process of clonal expansion of activated T, B, and NK cells. Moreover, the Th1 cytokines cause further recruitment of macrophages into the infection site [140], emphasizing Th1 hyperactivation and reinforcing gastric inflammation finalized to the decrease of bacterial density.

During the early phases of infection, T lymphocytes from the *H. pylori*-infected gastric mucosa are not able to secrete Th2 cytokines. Indeed, IL-4 from basophils and mast cells stimulates the expression of the Th2 cell-specific master transcription factor GATA-binding protein-3 (GATA-3) in TCR-engaged naïve T cells. GATA-3 have reciprocal antagonistic activity with T-bet, and both transcription factors are involved in attenuating the harmful effects of Th1 response to maintain an healthy homeostasis [141]. Moreover, *H. pylori*, through HP-NAP and other virulence determinants, plays a central role in inhibiting the pathway of Th2 differentiation, promoting IL-12 and IL-23 secretion by neutrophils and monocytes, which support the polarization of Th1 and Th17 against *H. pylori*, respectively. However, negative feedbacks down-modulating Th1 responses can be exerted by some *H. pylori* virulence factors and by components of the inflammatory milieu. For instance, bacterial molecules such as GGT or Lewis-antigens on LPS can activate tolerogenic DC subsets unable to foster a Th1 differentiation and response [142, 143]. In addition, IDO, high levels of COX-2 and prostaglandin-2 (PGE-2) modify the Th1/Th2 balance in favor of the Th2 response [144–146].

Th2

The Th2 cytokine profile includes IL-4, IL-5, and IL-13, which are involved in a paracrine and autocrine self-activation and self-maintenance circuit. These cell subsets are important for the production of *H. pylori*-specific IgG, IgM, and IgA, which intervene in systemic and local antibody-mediated protection against the bacterium. Especially IgA are relevant in inhibiting the bacterial colonization of the mucosa [147]. Th1 immune responses are more efficient than Th2 responses against bacteria [148], but, when mechanisms down-modulating Th1 expression occur, Th2 and Th17 seem to prevail, and an imbalance toward Th2 responses is shown. Patients with precancerous gastric lesions and gastric cancer express a predominant Th2 signature [149, 150]. One of the mechanisms which links Th2 profile to worse prognosis is represented by the ability of GATA-3 to down-modulate onco-suppressor genes [151].

T Regulatory (Treg)

Treg subsets, together with MDSCs, play a clincher role in *H. pylori* immune escape, since they can suppress DCs and effector T cells by cell to cell contact and production of TGF-β, IL-10, and IL-35, which limit the inflammatory responses. They are delegated to maintain self-tolerance and physiological conditions avoiding autoimmunity. Two kinds of Tregs have been described with different ontogeny but some common features: the natural Tregs (nTregs) and the induced Tregs (iTregs). While nTregs are generated within the thymus from lymphoid precursors, the naïve $CD4^+$ T cells residing in peripheral lymphoid organs and stimulated by the antigen can differentiate into iTregs in the presence of TGF-β and IL-2. Commonly both kinds of Tregs are defined by the intracytoplasmic expression of the transcription factor FoxP3.

Triggering of TLR-2 signaling pathway through *H. pylori* components LPS or HP-NAP is an important mechanism for Treg activation accompanied by Th1 inhibition. The Treg-induced onset of immunologically tolerant gastric microenvironment modulates the survival and persistence of *H. pylori* and directs the disease to a worse outcome [152, 153]. The increase in Tregs levels within gastric mucosa seems to be associated with increased expression of programmed death-ligand 1 (PD-L1) on epithelial cells in the site of infection. The binding of PD-L1 to inhibitory receptors present on the surface of $CD4^+$ T lymphocytes, such as PD-1 or B7.1, transmits inhibitory signals which reduce the effector capacity of these subsets [154]. Hence, globally, an immunological anergy is established in the field of infection and cancerization, favoring the immune evasion of the bacterium and transformed cells. It has been observed that *H. pylori*-induced DCs stimulate proliferation of Treg possessing a reduced suppressive function due to the *H. pylori*-dependent IL-1β secretion by DCs itself, suggesting an attempt to maintain or restore an inflammatory milieu with effector proprieties [155]. The transition to anergic or reactive immunity also depends on the balance between the signaling pathways conveyed toward Tregs or Th17 subset differentiation. In particular, the absence or the presence of IL-6, together with the activation of IL-6/STAT-3 axis in naïve CD4+ T cells, prompts or suppresses the expression of FoxP3, determining the fate toward the differentiation of suppressive or reactive T cells, respectively [156, 157].

Besides the essential immunological components described in this paragraph and their basic relationships participating to an evolving immune profile within the gastric precancerous lesion, other cellular subsets, such as Th9 and Th22, are strictly interrelated and committed in the progression/regression of the infection and of the field cancerization. They are elegantly reviewed elsewhere [102, 103]. Furthermore, host genetic factors related to immunological and regulatory elements composing the mucosal milieu and entangled in bacterial interactions may play a pivotal role in addressing the outcome of *H. pylori* infection.

Host Factors Affecting Inflammation and Its Clinical Outcome

Functional polymorphisms that influence the level or the quality of the expression of genes encoding for intracellular and extracellular receptors, enzymes, cytokines, and chemokines modulating the inflammatory response have been associated with increased risk of gastric cancer [158]. The clinical significance of these associations is dependent from ethnicity, which is an important confounding factor in epidemiological studies [159, 160]. Interestingly, a correlation between the presence of certain single nucleotide substitutions (SNPs) and a high proportion of highly virulent *H. pylori* strains has been found, suggesting the existence of a selective pressure exerted by the host on the microorganism subtypes. This possible synergistic interaction could lead to the progression or regression of precancerous lesions [20, 161]. Individual genetic predisposition to exacerbate or dampen the effects of *H. pylori* infection may concern several steps of the interplay between the bacterium and the host (Table 1.1).

Table 1.1 Some genetic polymorphisms involved in modulating host response during *Helicobacter pylori* infection

Host gene	Polymorphism	Associated clinical condition	Ethnicity	References
Mucosal invasion and damage				
PTPN11	rs2301756 A>G	Hp-related atrophic gastritis	Japanese; Japanese Brazilian	[162, 163]
	rs2301756 A>G	GC	Japanese	[164]
	rs12229892	GC and/or AG	Chinese	[165]
CDH1	rs16260 (−160 C>A)	GC	Caucasian	[166–172]
DNA repair				
PARP-1	rs1136410 T>C	Cardiac adenocarcinoma	Chinese	[173]
	rs1136410 T>C	High proportion of high virulence strains	Brazilian	[161]
DNMT	rs1550117 AA	GC	Chinese	[174]
Immune evasion and attenuation				
TLR1	rs4833095	Gastroduodenal diseases (including GC)	Malaysian	[175]
TLR4	rs4986790	Hp infection, AG, GC	Italian, Caucasian	[176, 177]
	rs4986791	Hp infection, AG, GC	Italian	[176, 178]
	rs4986790, −1	Digestive cancers	Caucasian	[179]
	rs11536889 G>C	Atrophy in Hp^+ pts	Japanese	[180]
TLR2	−196 to −174 del	GC; IGC, DGC	Brazilian; Japanese	[181, 182]
	–196 to −174 ins	Atrophy, IM	Japanese	[183]
TLR5	rs5744174 C	GC	Chinese	[184]
TLR10	rs10004195	Gastroduodenal diseases (including GC)	Malaysian	[175]
NOD1	rs2075820 AA	DU, atrophy, IM, Hp eradication failure	Hungarians; Turkish	[185, 186]
	rs2075820 AA	DGC	Chinese	[187]
	rs2075820 AA	Gastritis, ↑IL-8, and COX-2 mRNA	Korean	[188]
	rs2709800 GT	Gastric lesions; IM	Chinese	[189, 190]
	rs7789045 TT	GC	Chinese	[187]
NOD2	rs718226 G	Dysplasia	Chinese	[189]
	rs2111235 C	↑risk of disease progression in Hp^+ pts	Caucasian	[191]
	rs7205423 G	↑risk of disease progression in Hp^+ pts	Chinese	[187]
	rs7205423 GC	GC	Chinese	[187]
	rs2066842 (c.802CC>T)	GC	German, Polish	[192, 193]
	rs2066844 T	GC	Italian	[194]
	rs2066844 and rs2066845	↓autophagy and presentation to MHCII	Not specified	[195]
	rs2066847	↓bacterial clearance by monocytes	Italian	[196]
IL-1β	31C>T	GC, only in Hp^+ pts	Chinese	[197]
	511C/T	GC	Mixed; Caucasian	[19, 20, 198]
IL1-RN	IL1-RN*2 VNTR	GC	Non-Asian populations; Caucasian	[20, 199]

Table 1.1 (continued)

Host gene	Polymorphism	Associated clinical condition	Ethnicity	References
IL8	251 A/T	GC	Chinese; Asian	[200, 201]
IL10	1082 A/G	GC	Asian; Taiwanese	[199, 200, 202]
	819 C/T	GC	Taiwanese	[202]
TNF-α	308 G/A	GC	Caucasian	[203]
	238 G/A	GC	Asian	[204]

PTPN11 tyrosine-protein phosphatase non-receptor type 11, *Hp Helicobacter pylori*, *GC* gastric cancer, *AG* atrophic gastritis, *PARP-1* poly-ADP-ribose polymerase 1, *DNMT* DNA methyltransferase, *TLR* toll-like receptor, *pts* patients, *IGC* intestinal-type gastric cancer, *DGC* diffuse-type gastric cancer, *IM* intestinal metaplasia, *NOD* nucleotide-binding oligomerization domain-containing protein, *DU* duodenal ulcer, *IL* interleukin, *COX-2* cyclooxygenase-2, *MHCII* major histocompatibility complex II, *VNTR* variable number tandem repeat, *TNF-α* tumor necrosis factor α

Accumulation of DNA damage following oxidative stresses can be worsen by SNPs present in the host genes coding for DNA repair enzymes, which may unbalance the relationship between apoptosis and cellular proliferation. Poly-ADP-ribose polymerase 1 (PARP-1) is a component of the BER system whose polymorphisms have been mentioned to be associated with gastric cancer in some studies [161, 173]. However, some investigations report no relationship between worse prognosis and this mutated enzyme [205], while only combined effect of genetic and *H. pylori* profile covariates shows significant associations with gastric cancer in other studies [206].

One of the intracellular receptors which plays a pivotal role in the transformation of the infected cells is the tyrosine phosphatase Src homology region 2 domain-containing phosphatase-2 (SHP-2), which is first intercepted by the phosphorylated CagA and which was found to induce cell morphological and physiological modifications. SHP-2 is coded by the *PTPN11* gene, whose polymorphisms have been associated to increased risk of atrophic gastritis in Chinese population with, but not without, *H. pylori* infection. This effect is probably due to a different strength of signal transduction through the CagA-SHP-2 complex [165]. Although wide association studies focusing on hundreds of SNPs possibly involved in CagA interaction have identified new susceptibility loci for gastric cancer, the insufficient statistical power of these studies does not allow to assess the exact relationship between the selected SNPs and gastric cancer risk, providing only clues on the mechanisms entailing CagA function [207, 208].

Among polymorphisms concerning TLRs, two SNPs within *TLR4* coding gene have been linked with susceptibility to chronic infection, atrophic gastritis, and gastric cancer in Caucasian population by more than one study [176–178]; moreover, an alteration in the ligand-binding receptor site with proven diminished LPS responsiveness has been underlined [209, 210].

In addition to TLR, NLRs are important in the recognition of *H. pylori*. Polymorphisms of NOD1 and NOD2 are the best characterized in manifold studies. Overall, they highlight that functional SNPs reducing NOD1-/NOD2-mediated immune response to *H. pylori* contribute to bacterial survival and persistence and that a subsequent over-activation of other inflammatory responses may result in inflammation-related carcinogenesis [211].

As already mentioned, IL-1β is an important pro-inflammatory cytokine and a powerful inhibitor of gastric acid secretion, hence an inducer of atrophy progression. Polymorphisms in the *IL1B* promoter region, together with those concerning the IL-1 receptor antagonist (IL1-RN), have been reported to modulate IL-1β levels and action and be associated with an increased risk of gastric cancer [19]. These associations have been partially confirmed for Caucasian subjects by meta-analyses [212, 213], even if slightly contrasting results have emerged due to different grouping of

subjects with different allelic frequencies or different genetic models of analyses [214]. A meta-analysis including 36 studies to evaluate the effect of TNFA on genetic susceptibility to gastritis and gastric cancer has shown that the TNFA -308G>A polymorphism is a risk factor for developing gastric tumors in different ethnic groups, with significant results found in Caucasians, but no significant associations among East Asians or other ethnicities [159]. A meta-analysis on a total of 203 studies assessing associations between gastric cancers and 225 polymorphisms in 95 genes showed ambiguous effects for several gene polymorphisms between Asian and Caucasian populations. However, this study was able to confirm, through gene clusters, two panels of polymorphisms that were significantly associated with the risk of gastric cancer and able to specifically distinguish these two different ethnic groups [160].

The results of association studies between genetic determinants and *H. pylori*-related gastric carcinogenesis may suffer from bias linked not only to the selection of the analyzed subjects but also to the population sample size, to the interactions between several covariates that can have an impact on this system and cannot be all eligible or valuable, and to the intrinsic limitations of the statistical methods applied in these complex contexts. However, they may help in personalization of the surveillance if they are directed to specific patient populations.

Conclusions

Gastric tumorigenesis is a multifactorial process involving complex interactions between gastric microenvironment, inflammation, and colonizing microorganisms, with *H. pylori* being the most studied and well-known cancer determinant. In dependence on its genetic and phenotypic heterogeneity, *H. pylori* triggers a number of innate and adaptive immune responses entangled in tumor formation process. *Cag*A$^+$ strains present an increased risk of gastric cancer, and elevated levels of inflammatory cytokines have been observed in *H. pylori*-infected individuals. Through these mediators, several kinds of immune cells are stimulated to cooperate in the modulation of the oncogenic and anti-suppressive pathway activity. Methylation of tumor suppressor genes increases the risk of adenocarcinoma in the stomach. Autophagy and apoptosis processes may be hijacked toward cell growth and differentiation.

New technologies allow to discover additional elements, which can inflame the progression of the precancerous gastric lesions occurring in achlorhydria and atrophy settings. However, functional and mechanistic studies are needed to elucidate their specific activities within the evolution and dynamics of inflammation and their correlations with the pathogenesis of gastric cancer. Understanding of the mechanisms that regulate cancer-associated inflammation could open the way to new biomarkers able to distinguish patients with precancerous lesions that will remain indolent from those that will evolve, and to unexplored treatment opportunities influencing prevention and prognosis of therapeutic options.

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2 Genetic and Epigenetic Mechanisms in Gastric Cancer

Valli De Re and Riccardo Dolcetti

Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related deaths [1] and is a complex heterogeneous disease. Besides tumor node metastasis (TNM) staging, GC has two clinically accepted classifications based on histologic features: the Lauren's criteria, in which intestinal-type and diffuse-type adenocarcinomas are the two major histologic subtypes [2], and the World Health Organization (WHO) classification that differentiates GC into categories such as tubular, papillary, mucinous, and poorly cohesive, including signet ring cell carcinomas, plus uncommon histologic variants [3]. Both classifications enable a better understanding of the biology of the GC, but so far they had limited success in promoting the development of subtype-specific treatment options, due to the complex heterogeneity of the disease. More recently, genomic studies and comprehensive characterization of GC have confirmed this complex heterogeneity by providing further insights into the pathogenesis of GC, proposing genetic/molecular subclassifications of the disease and identifying new potential therapeutic targets. This may pave the way for the development of personalized prognostication and treatment [4].

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Etiological Classification

Diverging trends in the incidence of GC by tumor location and histology have suggested that GC heterogeneity may result from differences in the etiology. During the past two decades, while there has been a marked decline in distal and primarily intestinal type (mainly antrum and pyloric regions of the stomach) [5], the incidence of proximal diffuse GC type (the first three parts of the stomach, cardia, fundus, and body) has been increasing, particularly in the Western countries and Asia (particularly Japan, China, and Korea) (Fig. 2.1) [6–8].

Incidence by tumor sub-site also varies widely based on geographic location, race, and socioeconomic status. Distal GC predominates in the Republic of Korea, followed by Mongolia, Japan,

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_2

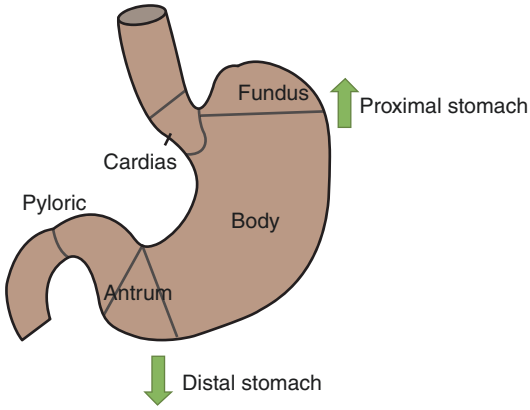

Fig. 2.1 The anatomical location of GC. During the last years, the numbers of proximal and diffuse types of tumors are increasing, while for the distal neoplasia, mainly of intestinal type, the number of cases is decreasing

and Colombia, and in lower socioeconomic groups, whereas proximal tumors are more common in developed countries, among whites, and in higher socioeconomic classes. The main risk factors for distal GC include *Helicobacter pylori* (*H. pylori*) infection and dietary factors, whereas gastroesophageal reflux disease and obesity play important roles in the development of proximal cardia/stomach cancer. Patients with immune deficiencies (i.e., immunodeficiency syndrome acquired and posttransplant immunodeficiency) have also an increased risk for GC [9]. Nonetheless, intrinsic genetic factors could play an additional key role in determining GC development since only a small part of *H. pylori*-infected individuals will progress to GC [10] and about 10% of cases occur in familial GC clusters with some cases showing specific germline mutations [11].

Inherited Genetic Predisposition

The first major inherited form of diffuse GC (HDGC) was found in linkage to the E-cadherin (CDH1) gene on chromosome 16q22.1 that encodes the epithelial cadherin protein (E-cad) (Fig. 2.2). CDH1 has been considered to be the prototypic gene of the cadherin family [14]. E-cad is a calcium-dependent cell-cell adhesion glycoprotein composed of five extracellular cadherin repeats, a single transmembrane domain, and a cytoplasmic domain with highly conserved binding sites for p120-catenin (also known as catenin-δ1) and β-catenin (Fig. 2.3). E-cad suppresses tumorigenicity and tumor dissemination by complex mechanisms that promote tissue organization and block of the apoptosis [16]. Moreover, the ectodomain of E-cad mediates bacterial adhesion to mammalian cells, and the cytoplasmic domain is required for bacterial internalization. Tumor pathogenesis are thought to involve biophysical adhesion processes and mechanotransduction-based intracellular signaling coupled to inhibition of molecules such as β-catenin and epidermal growth factor receptor (EGFR). EGFR belongs to a family of receptor tyrosine kinases that includes three other members (erbB2/HER-2, erbB3/HER-3, and erbB4/HER-4). These receptors are anchored in the cytoplasmic membrane and share a similar structure that is composed of an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and an intracytoplasmic tyrosine kinase domain (Fig. 2.3). Malignant carcinoma cells abrogate CDH1 function in numerous ways [16]. In HDGC cases, the CDH1 gene showed a damaging mutation leading to the production of a truncated or incorrect E-cadherin protein (E-cad) [17]. Patients had an autosomal-dominant inheritance and an earlier age at onset of the disease (<40 years, range of 14–69 years). Tumors were primarily of diffuse-type histology, a poorly differentiated adenocarcinoma that infiltrates into the stomach wall causing solidity of the wall (*linitis plastica*) without forming a distinct mass. Diffuse GC is also referred to as signet ring carcinoma with increasing proliferation, invasion, and/or metastasis. The estimated cumulative risk of GC by age 80 years is 80% for both men and women. Women also have a 39–52% risk for lobular breast cancer. Somatic mutations in CDH1 had been also reported in GCs, and lobular breast cancers that were not necessarily familial and CDH1 gene mutations also correlated with the risk of colorectal, thyroid, and ovarian cancer.

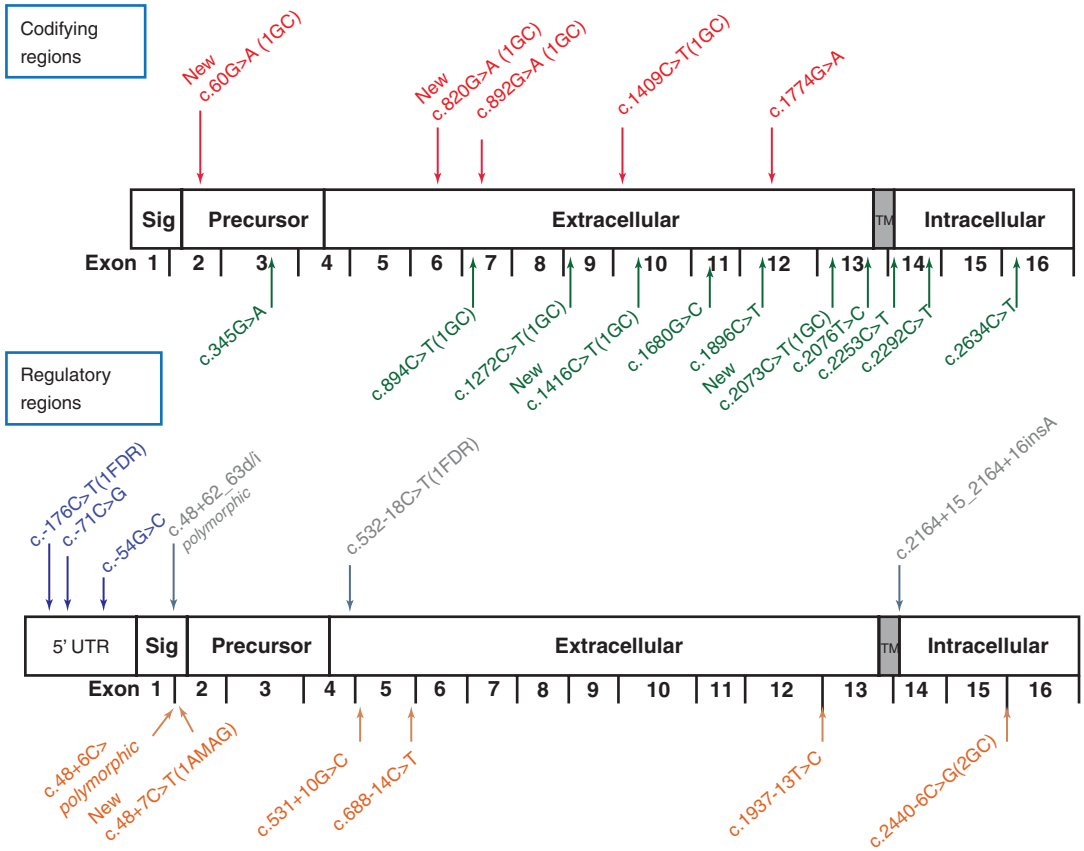

Fig. 2.2 CDH1 gene with some representative germline sense and missense mutations found in patients with gastric cancer [12]. The figure illustrates the high number of mutations, including new ones, their distribution along all the entire gene, and their potential impact on gene expression. Mutations had a different impact on the clinical status in patients, but only a few of them had a clear pathogenic role [13]

The HDGC syndrome was defined by the International Gastric Cancer Linkage Consortium (IGCLC) as the presence of two or more documented cases of diffuse GC in first- or second-degree relatives with at least one case diagnosed prior to age 50 years or three or more documented cases of diffuse GC in first- or second-degree relatives, regardless of age of onset [18]. Table 2.1 reports clinical criteria for the genetic screening of families with suspected hereditary gastric cancer according to the IGCLC guidelines updated in 2010.

Table 2.2 reports difference in the families with aggregation of GC and GC at early onset.

About 30–40% of HDGCs can be explained by defective germline alleles of CDH1, but for the remaining families, the factors driving susceptibility remain unknown even if in most cases, a reduced expression of the E-cad protein was present in the tumor tissue [20]. Of additional interest, in some cases of HDGC without CDH1 mutation, variants of genes encoding for CTNNA1, a truncated α-catenin [21], mitogen-activated protein kinase (MAP 3K6) [22], and insulin resistance receptor (INSR), FBXO24, and DOT1L [23], were discovered. Since α-catenin [24], MAP 3K6 [24], and INSR [25] function in complex with E-cad [25], genetic alterations of

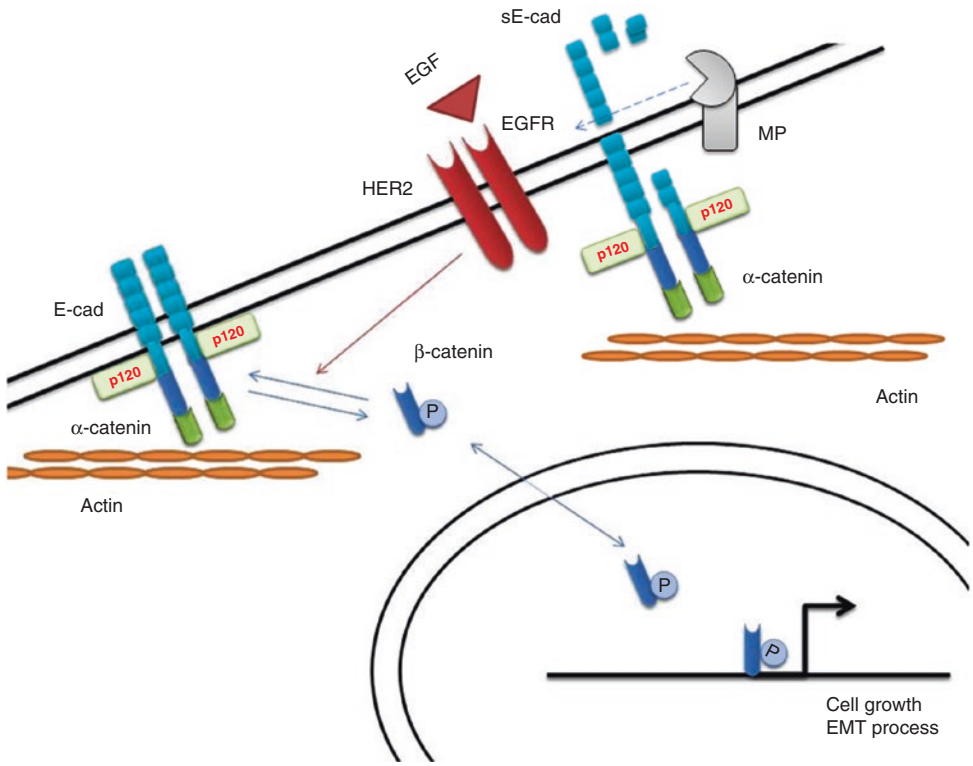

Fig. 2.3 Schematic diagram of E-cadherin-HER2 interaction. The mature E-cadherin contains three distinct domains: the highly conserved cytoplasmic domain, a single pass transmembrane domain, and an extracellular domain. The cytoplasmic tail of E-cadherin consists of two regions: the catenin-binding domain and the juxta-membrane domain. β-Catenin binds to the E-cadherin domain, and this complex via α-catenin connects and regulates E-cad interaction with the actin cytoskeleton. p120-catenin binds the CDH1 juxta-membrane domain and stabilizes E-cad expression at the cell surface. Activation of the HER2 receptor (e.g., by epidermal growth factor (EGF) ligation to the EGF receptor (EGFR) by inducing the phosphorylation of β-catenin directs the dissociation of β-catenin from the E-cad complex, thus leading to a decrease of E-cad-mediated cell adhesion, enhanced epithelial-mesenchymal transition (EMT), and increased translocation of β-catenin into the nucleus where it acts as a transcriptional regulator of genes involved in cell growth and EMT process. Metalloproteinases (MP) lead to production of soluble E-cadherin (sE-cad) through the cleavage of E-cad. HER2 phosphokinase activity favors the dissociation of β-catenin/E-cad complex leading to GC progression and metastasis. The production of sE-cad, as a paracrine/autocrine signaling molecule, not only undermines adherence junctions, causing a reduction in cell aggregation capacity, but its diffusion into the extracellular environment and the blood regulates multiple signaling pathways involved in GC progression [15]

Table 2.1 Criteria for the genetic screening of families with suspected HDGC; one of the following cases

Two GC cases in a family, in which one individual developed confirmed diffuse GC under age 50 years
Three confirmed individuals with diffuse GC in first- or second-degree relatives independent of age
One case of diffuse GC occurring before age 40 years
Personal or family history of diffuse GC and lobular breast cancer, one diagnosed before age 50 years

Table 2.2 Families with aggregation of GC and GC at early onset

Hereditary diffuse gastric cancer (HDGC): Families with aggregation of GC fulfilling the IGCLC criteria reported in Table 2.1
Familial diffuse gastric cancer (FDGC): Families with aggregation of GC and index cases with diffuse GC but not fulfilling the IGCLC criteria for HDGC
Familial intestinal gastric cancer (FIGC): Families with aggregations of GC and an index case with intestinal GC. No germline genetic defect has been found to date in this type of predisposing disease
GC at early onset: Patients who developed GC at an early age (<50 years old) without a familial history of GC [19]

these genes are particularly intriguing, but the exact contribution of these genes to GC predisposition remains unclear until a higher number of families with mutations in these genes will be reported and characterized.

Important observations in the last two decades allowed the identification of individuals who have inheredited a genetic mutation conferring susceptibility to syndromes including GC gene penetrance. While these individuals comprise a small portion of the overall burden of GC, the underlying inheredited genes identified are important to distinguish phonotypical features of GC and to better decipher the GC pathogenesis. Syndromes showing incomplete penetrance for GC include gastric adenocarcinoma and proximal polyposis of the stomach syndrome (GAPPS) [26], Lynch syndrome (or hereditary nonpolyposis colorectal cancer, HNPCC) [27], Li-Fraumeni syndrome (LFS) [28], hereditary breast and ovarian cancer [29], Peutz-Jeghers syndrome [30], and juvenile polyposis [24]. Of note, Asian ancestry of patients having one of these hereditary syndromes had showed a markedly increased risk of GC suggesting an interplay between genetic risk and environmental factors, e.g., *H. pylori* infection or food ingestion.

GAPPS is a phenotypic variant of the familial adenomatous polyposis (FAP) that is caused by a germline mutation in the 1B promoter region of the adenomatous polyposis coli (APC) gene. Interestingly, large deletions in the same APC gene region were observed both in families with more classic FAP phenotypes and in GAPPS, showing that APC promoter is a region of particular importance in gastric neoplasia [26].

The Lynch syndrome is a gastrointestinal disorder caused by germline mutation/deletion in one of the mismatch repair genes (MLH1, MSH2, MSH6, or PMS2) or in the EPCAM gene neighboring the MSH2 gene evidenced by gene microsatellite instability (MSI). EPCAM deletions cause additional methyl groups to be attached to the MSH2 promoter, thus reducing the expression of the MSH2 gene. GC is the third most common cancer in these individuals, with the intestinal subtype of antrum location being the predominant GC [31]. GC with MSI showed generally better survival rates, and it is particularly frequent in an area of Italy (Florence) with high GC risk [32].

The Li-Fraumeni syndrome is caused by germline mutations in the P53 tumor suppressor gene or the cell cycle checkpoint kinase (CHEK2) gene [31, 33]. Both diffuse and intestinal GC subtypes were observed.

Hereditary breast and ovarian cancer occurs in patients with germline mutations in the tumor suppressor genes BRCA1 and BRCA2. BRCA-related pathways safeguard genetic content such as DNA damage recognition, double-strand break repair, checkpoint control, transcription regulation, and chromatin remodeling; however despite the general nature of BRCA functions, tumors in mutation carriers predominantly target the breast and ovary. Some observational studies report elevations of the risk for certain cancers, including GC, besides breast or ovarian cancer in BRCA1 or BRCA2 mutation carriers [29].

Peutz-Jeghers and juvenile polyposis are two even more rare syndromes: the first caused by germline mutations in a serine/threonine kinase (STK11) [34] and the second one most frequently caused by mutations in the SMAD4 or BMPR1A genes [35].

Table 2.3 resumes the germline genetic alterations inheredited in syndromes predisposing to GC.

Table 2.3 Hereditary syndromes predisposing to gastric cancer

Syndromes	Gene inheredited
Hereditary diffuse gastric cancer [25]	CDH1
Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) [26]	APC
Lynch or hereditary nonpolyposis colorectal cancer, (HNPCC) [27]	MLH1, MSH2, MSH6, PMS2 or EPCAM
Li-Fraumeni (LFS) [28]	P53 or CHEK2
Hereditary breast and ovarian cancer	BRCA1 or BRCA2
Peutz-Jeghers	STK11
Juvenile polyposis	SMAD4 or BMPR1A

Genetic Alterations and Epigenetic Changes in Precancerous Lesions and GC at Early Diagnosis

In most instances, GC represents the culmination of a precancerous lesion sequence, i.e., metaplasia-dysplasia-GC [36]. Several comparative molecular genetics profiles of precancerous lesions and GC were performed to identify the genes and the mechanisms responsible for GC onset. Due to heterogeneity of the tumor, however, a unique panel for genetic alterations had not been found yet. However, epigenetic silencing of tumor-related genes by methylation (in particular, CDH1, runx3, MGMT, DAPK, CDKN2A, MLH1) and histone modifications were found to be restricted to cancer lesions and demonstrated to play an important role in GC pathogenesis [37]. Among these genes, MLH1 and CDKN2A presented a lower methylation frequency in intestinal metaplasia than in carcinoma suggesting a potential pathogenic role of progressively increasing levels of methylation in these genes. Methylation influences gene expression by affecting the interactions with DNA, proteins, and transcription factors. Promoter hypermethylation of the mismatch repair gene MLH1 is considered the main mechanism responsible for microsatellite instability in GC. Precancerous lesions are also characterized by a high frequency of hypermethylation of the CDKN2A gene, encoding for the cyclin-dependent kinase inhibitor p16, which slows down the cell cycle by prohibiting progression from G1 to S phase (Fig. 2.4) [38].

Hypermethylation of promoter CpG islands, which correlated with silencing of the downstream genes, reflects microsatellite unstable GC subtypes in *H. pylori*-positive [39] and EBV-positive GC subtypes [40].

More recently, an association between EBV-positive GC with aberrant histone modifications and related DNA methylation alterations has also been reported [40]. Histones are highly alkaline proteins with a high content in amino acids with basic side chains (particularly lysine and arginine). Their tasks are packaging and ordering of DNA into structural units called nucleosomes (Fig. 2.5). Nucleosomes represent the main protein components of chromatin, which is used to pack the large eukaryotic genomes into the nucleus, ensuring the appropriate access to DNA and correct gene expression. Posttranslational modifications of histones include acetylation/deacetylation, and methylation may thus interfere with gene expression.

Precancerous lesions often carry cyclin-E and cyclin-dependent kinase dysregulations (i.e., p15, p16, p21, p27) (Fig. 2.4) and alteration of the RAS-MAPK pathway and HER2 gene amplification (Fig. 2.6).

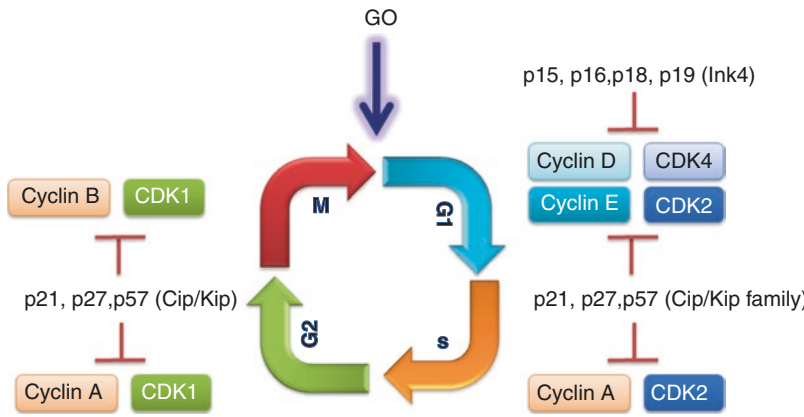

Fig. 2.4 Cell cycle progression. Orderly progression through the cell cycle involves passage through sequential checkpoints (i.e., G1, S, G2, M). Cyclin D1 binds to cdk4 and the assembly factor, p27, to create an active ternary complex. This complex can be inactivated by association with Ink4 or loss of cyclin D1 via proteasomal degradation. Through phosphorylation of intermediates, the complex induces genes involved in enhancing S-phase entry. The differential expression of cyclins and Cdks is highly coordinated and regulated through cell cycle progression

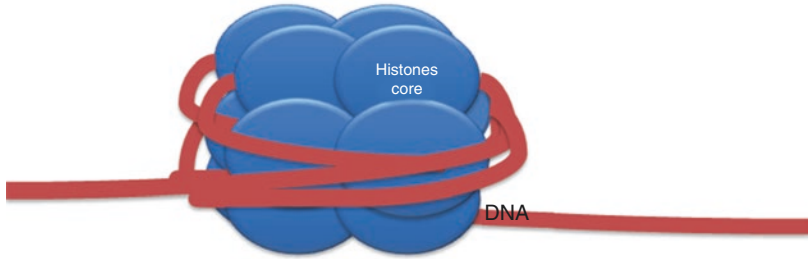

Fig. 2.5 Nucleosome is the basic unit of DNA packaging. It consists of a segment of DNA wound around a core of eight histone proteins. Histone modifications have a direct effect on nucleosome architecture. Posttranslational modifications of histones regulate DNA-templated processes, including replication, transcription, and repair. Acetylation, methylation, phosphorylation, and citrullination of the histone core may influence chromatin structure by affecting histone-histone and histone-DNA interactions

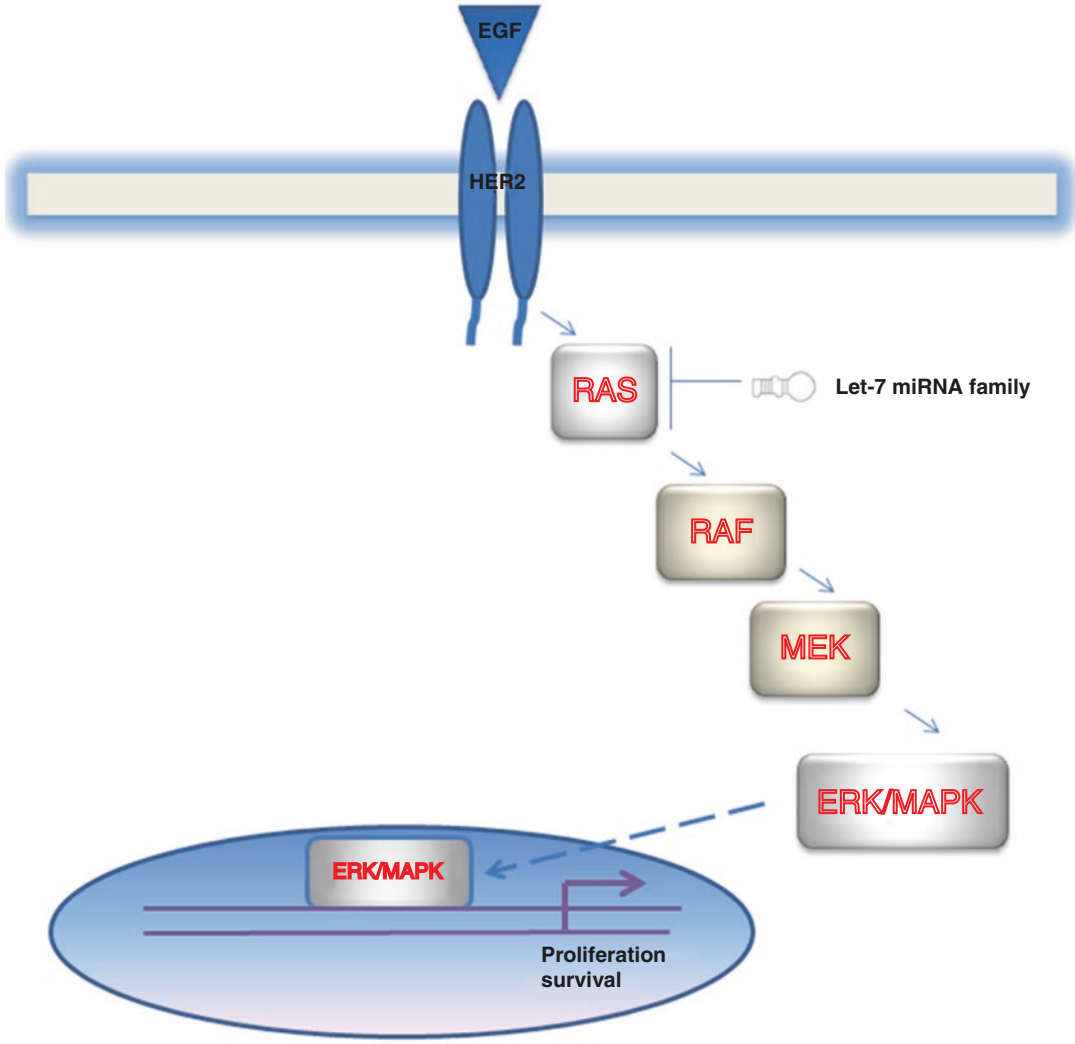

Fig. 2.6 RAS/RAF/MAPK pathway. Binding of a growth factor (e.g., EGF) to the tyrosine kinase HER2 activates the receptor activity. HER2 is activated either as a homo- or heterodimer and results in regulation of multiple pathways and in particular the RAS/RAF/MAPK pathway by downstream phosphorylation and activation of (H/N/K)-RAS, (A,B,C)-RAF, MEK1/2 (MAP 2K1), and ERK1/2 (MAPK1). Ultimately, ERK activation activates gene transcription that regulates cell proliferation and survival

Table 2.4 The most common molecular alterations found in precancerous lesions

Hypermethylation	MLH1 CDKN2A(p16)
Acetylation/deacetylation/methylation	Histone modifications
Gene dysregulations	Cyclin-E and cyclin-dependent kinases (i.e., P15, p16, p21, p27) RAS-MAPK pathway HER2 gene amplification
miRNA dysregulations	Let-7 family
Protein dysregulations	Tyrosine kinase (RAS)/HMGA2

An aberrant downregulation of microRNA expression, particular of the let-7 family members, has been observed in both gastritis and GC, especially during *H. pylori* infection [41, 42]. Importantly, their expression can be restored after *H. pylori* eradication [43]. Let-7 miRNA family is downregulated in various solid tumors and shows a key role in recognition of target oncogenic proteins such as the tyrosine kinase RAS (Fig. 2.4) and the high mobility group A2 (HMGA2), a non-histonic protein with structural DNA-binding domains acting as a transcriptional regulating factor.

Table 2.4 summarizes the most common molecular alterations often associated with premalignant lesions of the stomach and that become much more frequent in GC lesions, thus suggesting an association with malignant transformation.

Molecular Classification for GC

The first and most comprehensive molecular characterization for GC was proposed by the Cancer Genome Atlas (TCGA) project in 2014 [44]. Authors proposed a classification based on four subtypes: microsatellite instability-high (MSI-high), Epstein-Barr virus (EBV)-associated, chromosomal instability (CIN), and genomically stable (GS). The main distinctive characteristics of these subtypes are reported in Table 2.5.

The following year (2015), the Asian Cancer Research Group (ACRG work) provided a further molecular classification for GC, which also identified four subtypes with an increasing worse prognosis: microsatellite instability-high (MSI-high), microsatellite stable/TP53-positive (MSS/TP53-positive), MSS/TP53-negative, and MSS/epithelial-mesenchymal transition (MSS/EMT) [46]. ACRG introduced a role for P53, a key suppressor gene that responds to DNA damage and promotes apoptotic processes. Somatic P53 alterations have been reported in approximately 50% of overall human cancers [47]. The principal characteristics for ACRG subtypes are reported in Table 2.5.

ATCG and ACRG classifications showed a partial overlapping consensus such as microsatellite instability and EBV infection. However the ACRG is different for the demographic population and histologic subtype distribution, introduces the P53 mutations, and considers different baseline molecular mechanisms and prognostic factors [48]. Moreover, while microsatellite instability subtype showed a better prognosis in both these classifications, there were no prognostic differences in CIN and GS subtypes when TCGA classification was applied to the ACRG patient population. In addition, none of the two classifications takes into account the tumor microenvironment, the infiltrating immune cells, nor the role of tumor stage. Of note, both these molecular classifications failed to show significant survival differences in terms of OS or PFS when compared to simply staging tumor-nodes-metastases system (TNM – Union for International Cancer Control/American Joint Committee on Cancer) classification [49]. Thus, there is still an open debate about the reliability of current molecular classifications for GC prognosis. The use of more precise stratification criteria by combining TNM classification with histological and new molecular tools could achieve a more reliable classification impacting on therapeutic management of the patients in the near future.

Table 2.5 Molecular classification for GC

Classification	Subtypes			
ATCG	MSI (21.7%)	EBV (8.8%)	CIN (49.8%)	GS (19.7%)
	Prevalence female (56%) Older age at diagnosis (median age 72 years) Hypermutation (CpG island) MLH1 silencing Mitotic pathways	Prevalence in males (81%)[a] Fundus and body location (62%)[a] PIK3CA (80%), ARID1A (55%), BCOR (23%) mutations PD-L1/PD-L2 overexpression Hypermethylation CDKN2A (p16) silencing Immune cell signaling (Kak2, PD-1, PD-L) ARID1A and BCOR mutations	Intestinal GC Frequent gastroesophageal location (65%) TP53 mutation (71%) Receptor tyrosine kinase (RTK)/Ras amplification/activation (EGFR, HER2, HER3, VEGF-A, FGFR2, POCDILG2, PIK3CA, c-met) Amplification of cyclin genes (CCNE1, CCND1, CDK6). Constant activation of EGFR by phosphorylation/amplification	Invasiveness, diffuse histotype (73%) Younger age (median age 59 years) CDH1 (E-cad) (37%)/RHOA (15%) mutations CLDN18-ARHGAP fusion mutually exclusive with RHOA mutations Integrin and syndecan-1 pathway/angiogenesis
ACRG	MSI-high (22.7%)	MSS/TP53-positive (26.3%)	MSS/TP53-negative (35.7%)	MSS/EMT (15.3%)
	Intestinal histotype (60%) Antrum Location (75%) Hypermutation KRAS (23%), PI3K-PTEN-mTOR pathway (42%), ARID1A (44.2%), and ALK (16.3%) alterations	EBV Intact P53	Loss of functional P53 activity	Younger age Diffuse
	Best prognosis  Worst prognosis			

Cin chromosomal instability, *EBV* Epstein-Barr virus, *EMT* epithelial-mesenchymal transition, *GS* genomically stable, *MSI* microsatellite instability
[a]*MSS* microsatellite stable, *PD-L* programmed death ligand; brown color indicates an overlapping between the ATCG and ACRG subtypes [45]

At present, the possible advantage deriving from the use of a molecular classification is starting to become evident in both research and clinical settings. Encouraging positive results obtained with the use of immune therapies in MSI and EBV-positive GC subtypes and with two targeted agents, ramucirumab targeting VEGF [50, 51] and trastuzumab targeting HER2 receptor [52], are some important examples.

It is now well accepted that the presence of immune-active cellular components in the tumor microenvironment may contribute to a better prognosis in various tumors also including GC [53]. Tumors with high mutation burden or carrying mismatch repair deficiencies showed better and durable response rates after treatment with agents that control immune response [54, 55]. Inhibitors of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) receptor (pembrolizumab), or PD-L1 (avelumab), which restore T-cell activation, are now being used in several clinical trials [50]. Other agents targeting immune pathways are in clinical development (e.g., [56]). Notably, the load of tumor mutational burden was proposed as potentially suitable marker to predict the response to the anti-PD-1 treatment than expression of PD-L1 by immunohistochemistry [57, 58].

Available evidence indicates that EBV-positive GC, mainly associated with a MSI genotype, also showed encouraging response rates when treated with immune-based therapies [59]. EBV is a ubiquitous γ-herpesvirus distributed in the world's population with a high capacity in establishing immunoevasive latent infection (95%). EBV is also associated with mononucleosis and the development of both lymphoid and epithelial malignancies, particularly in immunodeficiency [60, 61] each characterized by a distinct pattern of viral protein expression [62–65]. Indeed during primary infection, EBV infects cells in a lytic form, and then the EBV genome is circularized, condensed, and methylated by host proteins to enter into a latent state in which only a small percentage of viral genes are expressed [66–70]. Several studies demonstrated that tumor cells of EBV-associated malignancies carry EBV as a latent infection [71–73]. It was then discovered that the switch of latent virus to lytic phase, leading to the expression of immediate-early, early, and then late protein through specific signaling cascade [74, 75], rendered the tumor cells more susceptible to the cytotoxic antiviral drugs and oncotherapies against EBV-associated malignancies such as GC [76–81]. In alternative, the discovery of lytic antigen (e.g., BARF1 antigen) abnormally expressed in EBV latent phase in some pathological situations including EBV-related nasopharyngeal carcinoma and GC could be also potentially appropriate targets for immune therapeutic treatment [82]. To identify GC subtypes that more likely respond to immunotherapy, a subclassification of GC has been recently proposed on the basis of PD-L1 expression, EBV status, MSI, and tumor-infiltrating lymphocytes (TILs) [83] or immune-related gene expression signatures, including interferon-gamma (6 genes) and expanded-immune (18 genes) signatures [84]. These findings are yet to be confirmed in prospective clinical trials.

The VEGF family consists of five ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor, PIGF) and three receptor tyrosine kinases (VEGF-R1,VEGF-R2, and VEGF-R3). VEGF is a signal protein that stimulates the formation of blood vessels (neoangiogenesis from pre-existing vasculature; vasculogenesis, for de novo formation), vasodilatation, and increased vascular permeability, overall mostly induced in hypoxic condition (release of HIF factor). All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors, such as the HER2 receptor (Fig. 2.7), and promoting their dimerization and the subsequent activation. Bevacizumab is the first anti-VEGF drug approved in 2004, while ramucirumab (AIFA 2014) is directed against the VEGF-R first FDA-approved therapy for advanced or metastatic GC after chemotherapy. Not all patients benefit from anti-VEGF treatment. Plasma VEGF-A and neuropilin-1 are emerging as potential predictive biomarkers for bevacizumab in GC, while biomarkers in patients treated with ramucirumab have yet to be identified.

HER2 is an oncogene encoded by the ERBB2 gene on chromosome 17. It belongs to the EGF receptor family and is overexpressed in 7–34% of GC. HER2 has no ligand-binding domain of

its own, but it does bind closely to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signaling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase (PIK3CA) [85] (Fig. 2.6). Alterations of HER2 structure, dysregulation of HER2 downstream signal effectors, and interaction of HER2 with other membrane receptors may interfere with the response to treatment [86]. E-cad/β-catenin (Fig. 2.3), RAS/MAPK (Fig. 2.6), and PI3K-Akt (Fig. 2.7) pathways are the main downstream signaling pathways of HER2. PIK3CA mutations and phosphate and tensin homolog (PTEN) inactivation were found to induce a hyper-activation of the PI3K-Akt pathway without the necessity of an upstream signal deriving from HER2 activation. It has now become clear that HER2 is expressed in many normal tissues, including the breast, gastrointestinal tract, kidney, and heart. Its major role in these tissues is to promote cell proliferation and suppress apoptosis, which may facilitate excessive/uncontrolled cell growth and tumorigenesis if aberrantly activated. Overexpression/amplification of HER2/*ERBB2* in breast cancer is associated with poor prognosis, increased risk of local recurrence, and distant metastasis. Conversely, the potential prognostic relevance of HER2 in the setting of GC is still inconsistent. Treatment leading to the selective inhibition of the HER2 protein has led to a modest survival benefit in GC; indeed, likewise HER2-positive breast cancer, patients are primary refractory or acquire resistance to trastuzumab therapy. Novel HER2-directed therapies including pan-HER TKIs, MET and mTOR inhibitors, and dual HER2-blockade are under investigations [87]. Specific targeted agents toward other genes/pathways with a key role in GC emerging from the ATCG and ACRG classifications (Table 2.5) are currently under investigation [48]. A list of the most promising targetable genetic lesions and signaling pathways is reported in Table 2.6.

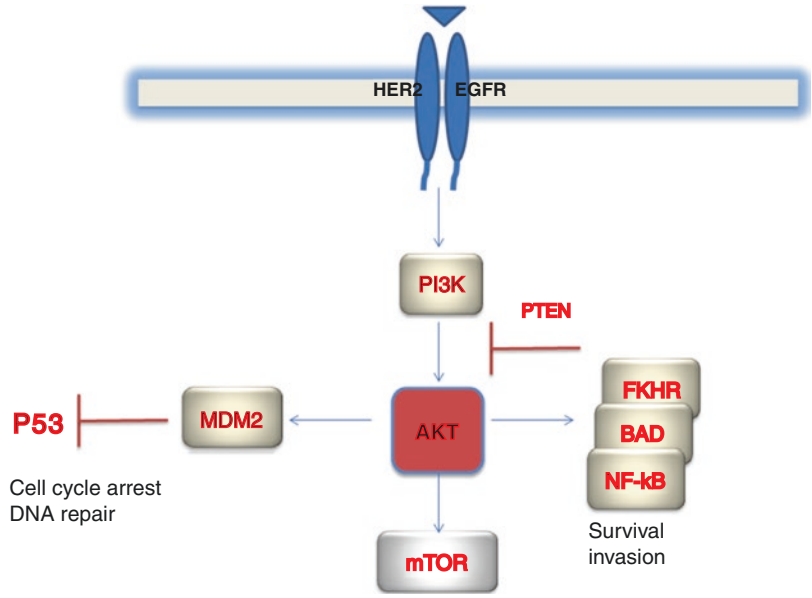

Fig. 2.7 Schematic representation of the PI3K/Akt pathway. HER2 activation leads to the activation of phosphatidylinositol-3 kinase (PI3K), which initiates activation of Akt by phosphorylation. Akt acts as a major source of activation to further downstream signaling genes involved in various cellular processes such as ribosomal protein synthesis and cell proliferation (through activation of mTOR), survival and invasion (through inhibition of BAD, FKHR and activation of NF-kB), metabolism (activation of GSK3β), cell cycle arrest, and DNA repair mediated by p53 (through MDM2)

Table 2.6 Emerging molecular markers and its targeted drug development

Gene	Activity	Positivity	Molecular alteration	Therapeutic agent
HER2	Member of the EGFR family, TYR kinase receptor	GC (7–34%) Intestinal (34%) Diffuse (6%) GJ (30%)	Amplification overexpression	Trastuzumab; other inhibitors had been tested: lapatinib, pertuzumab, trastuzumab emtansine; however benefit is modest. Resistance is under investigation
EGFR	TYR kinase receptor, most frequently form heterodimers with HER2	GC (24–27%) Intestinal (32.7%) Co-amplification (EGFR/HER2: 3.6%)	Amplification overexpression	Cetuximab; panitumumab. Disappointing results, but a possible lack of a proper selection of patients
P53	Cell cycle control, DNA repair, and apoptosis	GC (75%) Intestinal (50%) found also in adenoma and metaplasia	Mutation LOH	APR-246 and COTI-2 have progressed to clinical trials in some tumors
KRAS	RAS GTPase, recruits the cytosolic protein RAF	GC (5%) Intestinal (>50%) Associated with MSI	Mutation codon 12–13	No target therapies are currently approved. Other drugs, such as MEK (selumetinib), PI3K, or BCL-XL inhibitors, were tested in KRAS-mutated cancer cell lines with promising results
BRAF	Serine/threonine kinase	GC (2%)	Mutation, mostly V599M	Vemurafenib and dabrafenib have been approved for treatment of melanoma
FGFR2	Member of the fibroblast growth factor receptor family	GC (9%) Diffuse type (>50%)	Amplification	Several drugs and studies targeting this mutation are ongoing: AZD4547, dovitinib
MET	TYR kinase receptor, interacts with HGF (hepatocyte growth factor)	GC (8%) Diffuse (39%) Intestinal (19%)	Amplification Overexpression related to tumor stage and clinical outcome	Onartuzumab and an anti-HGF (rilotumumab) are studies discontinued (preliminary results were negative). By converse a positive tumor response to AMG337 was reported, but the study was interrupted for excess of agent toxicity. Study on LY2875358 is ongoing in other tumors
VEGF	Factor of angiogenesis	GC (50%)	Expression, prognostic for survival	Bevacizumab showed an improvement in progression-free survival and tumor response, but no overall survival benefit Ramucirumab and apatinib showed a significant improvement in the overall survival in subsequent line of treatments; their role in first-line therapy is still unclear
ATM	Serine/threonine kinase recruited and activated by DNA double-strand breaks	GC (13–22%)	Down expression Microsatellite mutation	Poli ADP-ribose polymerase inhibitor (olaparib)
Neo-antigens	MSI and MMR deficiency amplify the number of tumor neo-antigens	GC (30%)	Mismatch repair deficiency	Immunotherapies: pembrolizumab (PD-1); nivolumab plus/without ipilimumab preventive vaccine (against recurrent neo-antigens) is under study

Table 2.6 (continued)

CDH1	Tumor suppressor gene	GC (37% of GS subtype)	Mutations, hypermethylation downregulated expression	Treatments targeting epithelial-mesenchymal transition (EMT) are under study: emodin; an antimalarial agent ARS4, a steroidal alkaloid cyclopamine/ IPI-269609; a well-tolerated treatment for type 2 diabetes mellitus, metformin A clinical trial in hormone metastatic prostate cancer targeting E-cadherin is ongoing (NCT02913859)
ARID1A	Tumor suppressor gene involved in chromatin remodeling	GC (8%) GC (20% GS subtype) may synergize with PIK3CA activation and mutually exclusive to TP53 mutation	Inactivating mutations	Under study: EZH2, residual SWI/SNF activity, PI3K/AKT pathway, tumor immunological microenvironment, and stabilizing wild-type p53
RHOA	Ras-related family. It regulates cytoskeletal organization, cell adhesion, intracellular membrane trafficking, gene transcription, apoptosis, and cell cycle progression. Activates Stat3	GC (30% GS) Diffuse type	CLDN18-ARHGAP26 fusion gene or mutations	IMAB362 antibody against CLDN18-positive cancer. Fasudil, in other tumors
AURKA	Aurora family gene controlling mitotic events Serine/threonine kinase, located on centrosome	GC (5%)	Amplification, mutations	Alisertib
PLK1	Polo like kinase involved in the regulation of mitosis	GC (95%)	Overexpression	Volasertib
CLDN18	Member of claudins, components of the tight junction	GC (48%) Intestinal (>50%)	Downregulated Fusion gene with ARHGAP26, a gene encoding a RHOA inhibitor Mutually exclusive with RHOA mutations	Claudiximab (IMAB362)
MEK	Fibroblast growth factor receptor	GC (4%)	Amplification	Trametinib for treatment of melanoma BRAF+ GSK1120212 and PD0325901 in vitro
EBV	Epstein-Barr virus	GC (8.7%) Diffuse (50%)	Presence of the virus in the tumor cells	Immunotherapies PD1/PD-L1, JAK2 (pembrolizumab, nivolumab, MPDL3280A, MEDI 4736, AZD1480)

Abbreviation: *GJ* gastroesophageal junction, *MSI* microsatellite instability, *GS* genome stable ATCG subtype
Other drugs targeting PI3K/Akt pathway (4–24% of all GC, AZD5363, MK-226, BYL719) target of mTOR pathway (everolimus), ERB3 (15%, pertuzumab, trastuzumab)

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Part II

Clinical and Pathological Characteristics

3 Diagnosis and Surveillance: Endoscopic Hallmarks

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Introduction

There are some difficulties in early detection and diagnosis of stomach cancer because, usually, patients have non-specific symptoms as abdominal pain and a sense of fullness of the upper abdomen and in advanced stomach cancer poor appetite, weight loss, nausea and vomiting, and anemia. Patients presenting with the above-mentioned symptoms and patients with risk to develop gastric cancer require further workup [16]. Instrumental diagnostic tests, in patients with clinical symptoms or in presence of risk factors, include gastroscopy with biopsy, endoscopic ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), X-ray, laparoscopy and other laboratory tests [20]. Esophagogastroduodenoscopy (EGD) is the diagnostic imaging procedure of choice in initial step of diagnosis of gastric carcinoma (Fig. 3.1). An endoscope is used to visually examine the lining of the esophagus, stomach, and the upper portion of small intestine [6, 20]. EGD is performed with the patient in the left lateral position, usually under conscious sedation, mostly with benzodiazepines, sometimes in conjunction with a central analgesic and recently with propofol, and it is associated with very low complication rates [23]. After the general process of observation, differential diagnosis of minute mucosal changes found during the observational process should be conducted with caution by image-enhanced endoscopy (IEE). When judged to be necessary, the minimum necessary number of biopsy specimens should be obtained from the most suitable site. The presence of *H. pylori* infection, mucosal atrophy and intestinal metaplasia is closely associated with the risk of gastric cancer. Therefore, to recognize relevant endoscopic findings to these conditions, it is important to assess risk of gastric cancer and to detect early gastric cancer (EGC) efficiently [11]. The little adhesion of mucus, regular arrangement of collecting venules (RAC), and fundic gland polyps strongly suggest "*H. pylori* uninfected gastric

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_3

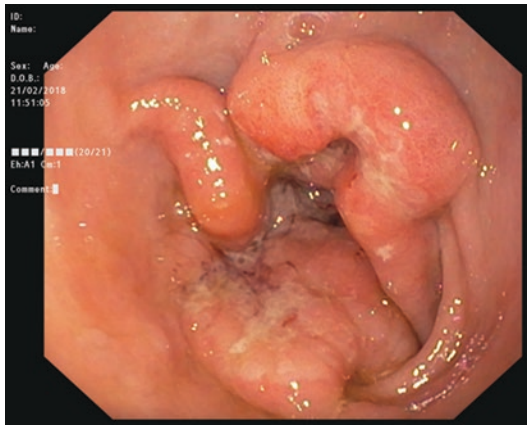

Fig. 3.1 Gastric cancer

mucosa." Atrophy of the gastric mucosa, meandering and thickening folds in the gastric corpus, xanthoma, or gooseflesh-like mucosa (nodular gastritis), indicates a "gastric mucosa with current or previous *H. pylori* infection" [11]. Undifferentiated carcinoma often originates from the region inside the endoscopic atrophic border or the intermediate zone (vicinity of the atrophic border). Well-differentiated carcinoma often arises from the external region of the endoscopic atrophic border.

The macroscopic aspect of gastric cancer is defined by Borrmann classification: polypoid lesions (type I); fungating, ulcerated with sharp raised margins (type II); ulcerated with poorly defined infiltrative margins (type III); and infiltrative, predominantly intramural lesion, and poorly demarcated (type IV).

EGD is a highly sensitive and specific diagnostic test, especially if combined with endoscopic macrobiopsies and histological examination of the tissue. The update Sydney system recommends at least five biopsies, two from the antrum, two from the corpus, and one from the incisura angularis, and multiple biopsies should be obtained from any suspicious areas. Gastroscopy must be performed with quality criteria, applying coloring methods (chromoendoscopy, NBI, etc.), magnification and endomicroscopy, which allow for high accuracy of diagnosis. There is no universal standard for the number of images to be recorded during EGD. The ESGE guideline recommends four images to be recorded for observation of the stomach [3]. This number is inconceivably low in comparison with the number of images usually taken in Japan. However, considering the low prevalence of gastric cancer in Europe, this number, reflecting moderate attention, may be appropriate and cause no clinical problems [11].

Recommendations for improving upper gastrointestinal (UGI) endoscopy in Western countries are:

- Focus training on early upper gastrointestinal cancer detection.
- Routine systematic mucosal washing with mucolytic and antifoam agents with or without use of antiperistaltic agents.
- Appropriate sedation to allow adequate examination.
- Systematic examination of upper gastrointestinal tract with routine high-definition white light photodocumentation using European Society of Gastrointestinal Endoscopy 2016 guidelines as a minimum standard.
- Minimum total procedure time 8 min: with 4 min gastric examination and 2 min oesophageal examination where detection of early upper gastrointestinal cancer is a diagnostic aim [31].
- When a gastric cancer is found, 6–8 biopsy specimens with macroforceps are recommended.
- This number of specimens provides a more correct evaluation of HER2 status.
- Further staging with endoscopic ultrasound in esophagogastric junctional tumors and selected gastric cancers is recommended (grade B) [2].

Chromoendoscopy and Magnification Endoscopy

Magnifying endoscopy in conjunction with chromoendoscopy is useful to improve visualization of mucosal details. Vital dyes in digestive endos-

copy have been introduced 20 years ago for a better identification of mucosal surface abnormalities. In recent times, vital dyes can be associated to endoscopic high resolution and image magnification tools (zoom endoscopies).

The most commonly used dyes are methylene blue and contrasting dyes such as carmine indigo. Methylene blue is taken up by absorbent tissues such as the small intestinal epithelium; instead carmine indigo is not absorbed from cells but has the objective of delineating the edges and contour of a lesion accurately, to facilitate their detection.

Endoscopic magnification, with tools that enlarge the image up to 150 times with the use of electronic zoom, has allowed to improve the identification of preneoplastic lesions and early neoplasia (in particular non-protruding and small ones). The use of this technique permits to reduce the rate of lesions not detected during traditional endoscopy and to study, analyzing spatial arrangement of glandular crypt orifices (pit pattern), the histological type of the lesion (hyperplastic or adenomatic), and the depth of parietal invasion. Moreover, chromoendoscopy with methylene blue or acetic acid allowed the development of some superficial classifications that correlate with intestinal metaplasia and dysplasia. Regular or destructured patterns are related to the presence of high-grade dysplasia or carcinoma [14].

Narrow-band Imaging NBI

Narrow-band imaging (NBI) is based on the shrinking spectral bandwidth of RGB optical filters used in the sequential imaging method that creates video-endoscopic images. The NBI system is embedded in the endoscope and filters some wavelengths allowing only blue light to illuminate the tissue thus permitting an increased vascular-capillary network visibility and to process the endoscopic image in real time (Fig. 3.2). In this way, the endoscopist can evaluate the capillary pattern correlated with the degree of parietal infiltration of the neoplasm. This technology, with endoscopic magnification, can identify capillary or glandular mucosal alterations.

Other innovative technologies even if of more limited use are autofluorescence (AF), exogenous fluorescence or photodynamic diagnosis (PDD), reflection or light scattering spectroscopy (LSS), trimodal spectroscopy, Raman spectroscopy, and optical coherence tomography [14].

Finally, a very important point is the introduction of endoscopic macrobiopsy. The use of macrobiopsy, in addition to the electronic technology, allows taking tissue samples of about 0.5–0.7 cm against the "normal" pliers with 0.2–0.3 cm, reducing the number of inadequate samples for histological diagnosis.

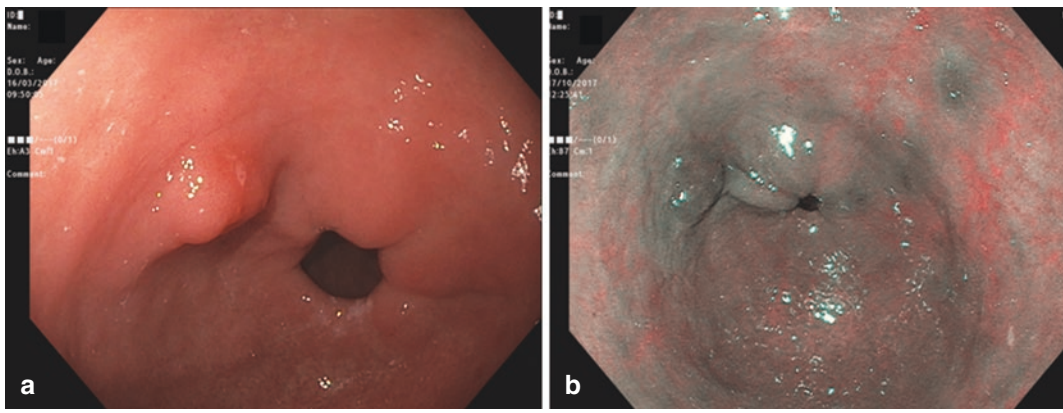

Fig. 3.2 (**a**) Endoscopic image of high-grade dysplasia; (**b**) NBI image of high-grade dysplasia

Confocal Laser Endomicroscopy

The confocal laser endomicroscopy (CLE) (CellVision, Mauna Kea Technologies, Paris, France) is a new endoscopic technique that is performed during a traditional endoscopic examination. It allows to examine mucous membranes and tissues during the diagnostic phase [14]. The confocal laser endomicroscope permits in vivo analysis of tissue microarchitecture, with cellular resolution, thus allowing a precise identification of areas to be biopsied. The analyzed region is enlarged 1000 times, so it permits to examine the mucosa and its glands, vessels, and microvessels at the same time and to interpret whether the alterations are inflammatory, preneoplastic, or neoplastic.

These features make pCLE potentially useful in early diagnosis of tumor or dysplastic lesions, as well as in biopsy optimization and targeted endoscopic treatment. The CLE probe is introduced into traditional endoscope during endoscopic examination. Endomicroscopic images are generated by the use of the contrast agent sodium fluorescein administered endovenously. After intravenous administration of 5–10 ml of 10% fluorescein sodium, cells, vascular system, and connective tissue can be well differentiated. During the acquisition of endomicroscopic images, the terminal of the probe should be gently rested on the mucosa/lesion to be investigated. 0.8 or 1.6 endomicroscopic images per second are displayed on the monitor and can be recorded in the database of the equipment.

The main indications to the endomicroscopic study are all those conditions in which it is possible to recognize alterations of cellular morphology or of vasculature in the superficial layers of the mucous membrane, especially the dysplastic lesions of the gastrointestinal tract, including the biliary duct.

Several clinical studies based on comparison with traditional histological examination have established the diagnostic confocal criteria for the diagnosis of normal gastric mucosa, chronic gastritis with intestinal metaplasia, and neoplasia. In the absence of pathology, the administration of fluorescein allows to identify in the gastric corpus a network of subepithelial honeycomb capillaries surrounding gastric foveole, while in the antrum, they have a spiral appearance. Early neoplastic well-differentiated lesions appear generally hypervascularized, with tortuous and dilated vessels with irregular form and dimensions. In contrast, undifferentiated tumor appears hypovascularized, and vessels have short and unconnected branches [8].

In a monocentric study conducted on 31 patients with 35 lesions, diagnostic accuracy of endomicroscopy was significantly higher than the histological diagnosis performed on standard biopsies (94% vs 86%), when the results were compared with the histological outcome of the entire post-ESD lesion. In the gastritis associated with the presence of *Helicobacter pylori*, CLE has demonstrated the presence of fluorescein outbreak through intercellular spaces. The eradication treatment reduced the fluorescein spill restoring the normal condition. Instead, the spill of contrast media persisted in the presence of morphological alterations, such as intestinal metaplasia, despite the success of eradication therapy. In this context CLE has highlighted the altered function of the in vivo mucous barrier, a factor that can contribute to carcinogenesis. Recently, due to the increasing interest in molecular imaging, specific biomarkers, also called molecular probes, have been developed. Typically, these are low molecular weight peptides, with variable affinity for specific structures, conjugated with fluorescein (e.g., fluorescent antibodies to the epidermal growth factor receptor – EGFR).

These antibodies allowed in vivo study of gastric cancer and possible response to targeted therapies in animal models and ex vivo on human tissues opening the way for new studies of markers that allow targeted use of drugs such as trastuzumab [13, 18].

Endomicroscopic observation is, however, time-consuming. Peristaltic visceral or transmitted (breath, heartbeat) movements and the remarkable enlargement of vision can generate

artifacts. The depth of exploration, limited to a maximum of 250 microns, does not allow to evaluate the neoplastic infiltration of the submucosa.

A particular field of endomicroscopy concerns the study of tumor neoangiogenesis. The development of new blood vessels from pre-existing vessels (angiogenesis) is a phenomenon indispensable both in normal conditions and in pathological situations such as growth and tumor progression. Tumors cannot grow more than 2 mm unless they are in presence of an angiogenetic process. Be able to identify the onset in a relatively short time of new vessels in the intratumoral area can be crucial for a decisive and personalized anti-angiogenetic therapy.

The fluorescein as a contrasting medium is very useful in highlighting these neoformed vessels that often exhibit large structures and with defective flow and leakage areas (Fig. 3.3). Spessotto et al. demonstrate that in a total of 35 consecutive patients with gastric cancer that underwent endoscopy and pCLE during the same examination, the morphological neoangiogenesis was in agreement with histological and immunohistochemical analyses. They develop an arbitrary angiogenesis scale that can estimate the extent of intratumoral angiogenesis based on vessel shape and size, permeability, and blood flow and allowed the creation of an angiogenic score ranging from 0, for normal vasculature, to 4, for aberrant vasculature.

The study shows that the angiogenic score may be applied during endomicroscopy with a moderate grade of "consistency," at least for rectal cancer patients, thereby granting very rapid information on the vascularization pattern of a given patient. A lower concordance related to gastric cancer analyses could be due to the excess of fibrotic tissue in gastric tumors, which may render difficult the clear detection of the vascularized regions by pCLE in real time. They overcome this problem by off-line evaluation since the dedicated software allows the images to be corrected and stabilized after digital storage. In any case, they demonstrate that off-line evaluation can provide information more rapidly than histological procedures [28].

Endoscopic Ultrasound

Endoscopic ultrasound (EUS) is a modality that allows more accurate locoregional staging of early or locally advanced gastric cancer. The transducer is placed directly next to the gastric wall, so the depth of tumor invasion and local lymph node involvement, that usually influence survival, can be determined by high-frequency soundwaves [16].

EUS imaging is currently performed with radial or linear echoendoscope. These scope are video-endoscope coupled to electronic ultrasound processors for generation of electronic EUS images, endowed with special aspect including Doppler, contrast, and others: standard EUS usually utilizes high ultrasound frequencies that vary between 5 and 20 MHz [33] (Fig. 3.4). The transducer in most radial echoendoscopes generates radial images of 360°, oriented perpendicular to the shaft axis of the instrument. Indeed, linear echoendoscopes produce images directed parallel to the tube axis allowing for an effective

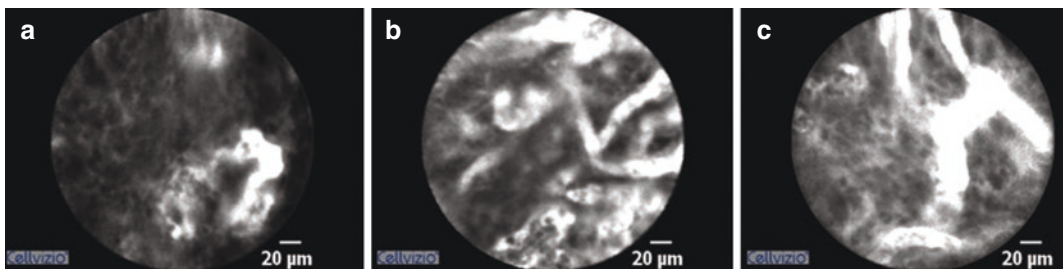

Fig. 3.3 Image of vasculature in gastric cancer obtained by probe confocal laser endomicroscopy

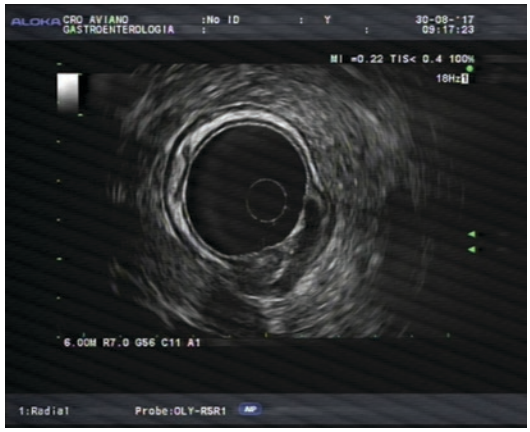

Fig. 3.4 Endoscopic ultrasound image of gastric cancer T3N0

and safe performance of EUS-guided fine needle aspiration puncture (EUS-FNA) when needed [33]. Acoustic coupling of the ultrasonic transducer to the GI wall requires application of fluid as interface between the wall and transducer. Usually water-filled balloon, placed around the tip of instrument, or instillation of water in the lumen is used to perform EUS [23, 33].

EUS can increase preoperative staging accuracy, but it cannot be used to assess distant lymph node involvement or to screen for lung or liver metastasis. EUS is useful in defining proximal and distal extent of the tumor and to evaluate T and N stage but is less useful for antral tumors [5, 27].

During echoendoscopy some scanning principles should be performed as scanning of target should be perpendicular to avoid erroneous diagnoses or overstaging due to broadening and blurring of structure; it should be kept an adequate focal distance; the use of higher frequencies may help to obtain a better visualization of structures and lesions [33].

The gastric wall normally consists of five distinct layers. The two inner layers (echo rich and echo poor) represent the interface/superficial mucosa and deep mucosa/muscularis mucosa. The third (echo rich) layer corresponds to the submucosa, the fourth (echo poor) to the muscularis propria, and the fifth (echo rich) to the serosa, which is difficult to distinguish from the surrounding tissue. For the orientation and other diagnostic purposes surrounding organs, vessels and other structures are very important [33].

EUS accuracy in determining infiltration degree of the wall ranges from 67% to 92%. A recent systematic review with meta-analysis has shown that the ability to accurately study gastric wall with EUS in cancer has a high accuracy compared to TAC or MRI in particular in the T1 and T4 stage, discriminating patients to be endoscopically resected and those in which surgery has little chance of treatment [17, 26].

Criterions to distinguish malignancy on EUS include hypoechogenicity, round shape, smooth, distinct margin, and size >1 cm [27].

EUS allows to perform a fine needle aspiration (FNA) or targeted needle biopsy on the suspicious lymph nodes.

Moreover, it has been demonstrated that EUS is useful in selecting patients who should undergo diagnostic laparoscopy; in fact, patients with EUS T1-T2, N0 staging have a 4% risk of peritoneal metastasis compared to the 25% risk in patients staged as T3-T4, N +, indicating how laparoscopy could be spared for subjects with EUS staging up to T2, N0 (negative predictive value of M1: 96%) [7, 22, 25]. Finally EUS is helpful in the diagnosis of linitis plastica.

Early Gastric Cancer

The Endoscopic Diagnosis of Early Gastric Cancer

The endoscopic diagnosis of early gastric cancer (EGC) requires good endoscopic techniques and thorough knowledge. The accuracy of endoscopy in the detection and diagnosis of EGC is reported to range between 90% and 96% [12]. Chromoendoscopy and magnifying endoscopy are promising image-enhanced endoscopic techniques for characterization. Early gastric cancer is defined as confined to the mucosa or submucosa, regardless of lymph node metastasis. To have an accurate diagnosis of early gastric cancer, it's very important to have a good knowledge of the characteristic of early-stage disease.

EGC can be divided into three types: elevated, superficial and excavated. The superficial type is further subdivided into superficial elevated, superficial flat and superficial depressed [32].

It is difficult to find superficial flat lesions in the conventional white light endoscopy (WLE), which often cause misdiagnosis and missed diagnosis. The most common lesions of EGC were usually manifested by erythema and erosion [34]. During white light endoscopy, it's important to pay attention to changes in color of mucosa (pale redness or fading of color), loss of visibility of underlying submucosal vessels, thinning of and interruptions in mucosal folds and spontaneous bleeding [32].

Most elevated EGCs are of the differentiated type, and some gastric superficial elevated type EGCs and adenomas appear whitish. Among the flat or depressed type EGCs, differentiated-type cancers look reddish, whereas undifferentiated types appear whitish because of a difference in hemoglobin content [11].

Chromoendoscopy

Several reports describe the magnification findings of early gastric cancer. The characteristic patterns of EGC are as follows: (i) a small regular pattern of sulci and ridges, (ii) an irregular pattern of sulci and ridges and (iii) a lack of visible structure. The presence of irregular minute vessels and the variation in the caliber of vessels are specific vascular patterns in EGC [4].

When mucosal changes are observed, chromoendoscopy can effectively aid to diagnosis. After spraying dye in lesion and over the mucosa surrounding the lesion, early gastric cancer is diagnosed through the comparison between the two parts. The detection of an irregular shape and distribution of microvessels make the difference between early cancer and focal gastritis. Irregular microvessels are tumorous vessels. The demarcation line between cancer and normal mucosa allowed the evaluation of the margin of the carcinoma before endoscopic resection [4].

It's important to wash the lesion accurately prior to spraying because the dye can make the lesion boundaries unclear when mucous is adherent to stomach wall. However, it's difficult to diagnose correctly gastric cancer smaller than 5 mm or superficial flat (IIb) gastric cancer using white light imaging or chromoendoscopy.

Narrow-band Image-Enhanced Endoscopy (NBI)

It enhances the superficial surface structure and vascular architecture of the mucous layer by illuminating blue and green narrowband lights. NBI is a promising technique for characterizing small or flat early gastric cancers. Microvascular and microsurface patterns on the gastric mucosa can be observed with NBI. Moreover it further reveals intestinal metaplasia by its whitish color. Intestinal metaplasia exists as a flat mucosa with subtle discoloration. 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. The "light blue crest" is thought to be caused by the reflection of short wavelength light at the brush border on the surface of the intestinal metaplasia [11].

Ultrasound Endoscopy

EUS can be used to make a more objective diagnosis. Through this diagnostic method, endoscopist can determine whether the patient can undergo endoscopic therapy and small diameter lesion is often targeted.

Therapeutic Endoscopy

Therapeutic endoscopy plays a major role in the management of gastric neoplasia. It is a local treatment of primary lesions, and it is totally ineffective if any metastatic lesions are present. It's indicated in cases of early gastric cancer if there are no lymph node metastasis [29]. Its indications can be broadly divided into four categories: to remove or obliterate the neoplastic lesion, to palliative recanalization of luminal obstruction, to

treat bleeding and others [9, 10]. Two techniques are used to treat endoscopically early gastric cancer: endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD).

Endoscopic Mucosal Resection

Mucosectomy or endoscopic mucosal resection (EMR) is a minimally invasive technique safe, convenient and efficacious for T1 mucosal cancers [10, 21]. EMR is used in alternative to surgery in well-differentiated elevated lesions, intestinal type adenocarcinomas with no ulcer, confined to the mucosa, smaller than 20 mm in size with no lymphatic or vessel involvement [9, 10, 21]. Endoscopic resection is comparable in many aspects to conventional surgery, with the advantages of being less invasive, more economical [10] and to permit a complete pathological staging of the cancer. The risk of lymph node metastasis can be predicted, after endoscopic resection, thanks to the pathological assessment of cancer invasion depth, of cancer differentiation degree and of lymphovascular invasion extent [10]. After the submucosal injection of the lesion, a specialized crescent-shaped snare is deployed in the groove at the tip of the cap. The snare is closed and resection is performed by electrocauterization and then the lesion is drawn into the cap connected to the tip of a standard endoscope. Caps are available in different sizes according to the diameter of the endoscope and the size of the lesion [9, 10, 30]. Another EMR technique is the ligation EMR that uses ligation devices to capture the lesion and transform it into a polypoid lesion deploying the band underneath it [9].

Endoscopic Submucosal Dissection (ESD)

A subsequent technique developed in therapeutic endoscopy, called endoscopic submucosal dissection (ESD), allows the direct dissection of the submucosa and the resection en bloc of large lesions [9]. ESD is performed with special endoscopic knives and permit the en bloc resection with a standard single-channel gastroscope [9]. The lesions that should be considered for endoscopic resection which are at very low risk of lymph node metastasis are the following: noninvasive neoplasia (dysplasia) independently of size; intramucosal differentiated-type adenocarcinoma, without ulceration (size $\leq$2 cm absolute indication, >2 cm expanded indication); intramucosal differentiated-type adenocarcinoma, with ulcer, size $\leq$3 cm (expanded indication); intramucosal undifferentiated-type adenocarcinoma, size $\leq$2 cm (expanded indication); and differentiated-type adenocarcinoma with superficial submucosal invasion (sm1 $\leq$ 500 μm) and size $\leq$3 cm (expanded indication) [24]. The three are the steps involved in ESD technique: fluid injection into the submucosal layer to separate it from the muscle layer, circumferential cutting of the mucosa surrounding the lesion and finally submucosal dissection of the connective tissue of the submucosa under the lesion [9].

Surveillance

Approximately 40–60% of patients treated surgically develop a relapse, and in 80% it will happen within the first 2 years. Regional site relapses occur in 20–30% of cases, while the liver and peritoneum are the long distant organs that frequently show recurrence.

The risk of relapse at 5 years is lowered to 47% in patients who survived a year from the disease and 10% in patients who survived 5 years.

A regular follow-up may allow investigation and treatment of symptoms, psychological support, and early detection of recurrence, though there is no evidence that it improves survival outcome. Follow-up should be tailored to the individual patient and the stage of disease [27]. To date, there are no randomized controlled trials in gastric carcinoma that may indicate appropriate follow-up of patients after surgical resection or after treatment (Level of Evidence 3) [1]. The main purposes of a follow-up strategy are the early detection of anastomotic recurrences that can be treated surgically, the assessment of

abnormalities concerning nutritional status (anemia, dumping syndrome), or identification of clinical signs related to recurrence. Given the lack of a significant impact on patients' survival using a regular follow-up of imaging, the international guidelines propose a clinical follow-up consisting only of the hematochemical parameters, leaving the instrumental investigations in relation to the symptomatology reported by the patient. In case of clinical suspicion of recurrence, CT appears to have higher sensitivity than ultrasound examinations (Level of Evidence 3).

The following scheme may be suggested:

- Every 3–4 months for the first 2 years (0–2 years): clinical examination including weight, blood tests (hemoglobin levels, sideremia, renal, and hepatic function), and instrumental to be performed on clinical need at the doctor's discretion.
- Every 6 months in the following 3 years (3–5 years): clinical examination including weight, blood tests (hemoglobin levels, sideremia, renal and hepatic function), and instrumental test to be performed on clinical need at the doctor's discretion.
- EGDS appears useful in particular in the case of subtotal gastrectomy for the search for local recurrences or cancer on the stump; it could be repeated every 2–3 years in the first 5 years and then every 3–5 years.

Although there are no published data, it is considered acceptable that after 5 years of specialist follow-up, surveillance may be continued annually, possibly by the general practitioner [15, 19, 27] (AIOM guidelines, 2015).

Acknowledgments The authors thank Francesca Masutti for her assistance in manuscript editing.

This work was supported by the following grant of Ministry of Health (*RF-2016-02361525* to R.C.).

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4 Pathological Diagnosis and Classification of Gastric Epithelial Tumours

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Precancerous/Early Cancerous Lesions

Intra-epithelial Neoplasia/Dysplasia

The multistep process of gastric carcinogenesis has been postulated by Correa [1] as a sequence of events, referred to as Correa cascade, where dysplasia or intra-epithelial neoplasia represents the penultimate stage of sequence [2] (Fig. 4.1).

In tumour pathology, dysplasia is a term that, literally, means abnormal growth. During the years, disagreements between American, European and Japanese pathologists lead to develop several classifications to standardize the definition of gastric dysplasia and neoplasia [3–5].

Nevertheless, despite the terminological differences between Western and Japanese pathologists, interpretative problems, including the distinction from inflammatory-related reactive or regenerative

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© Springer Nature Switzerland AG 2019
V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_4

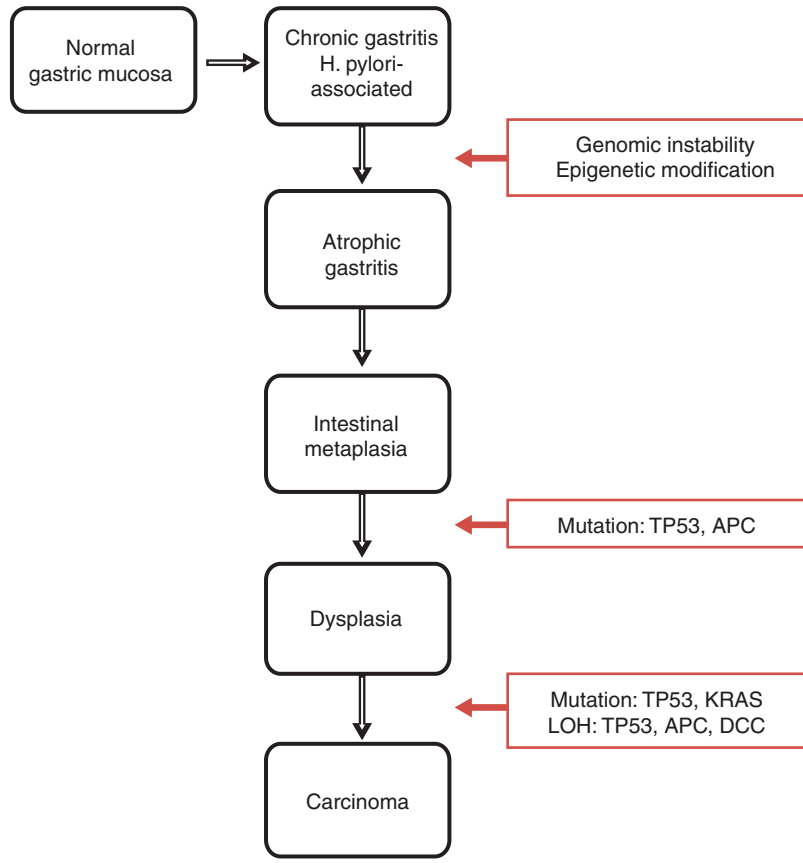

Fig. 4.1 The Correa cascade of gastric carcinogenesis

changes and the distinction between intra-epithelial and invasive carcinoma, limit the formulation of a correct diagnosis in grading gastric dysplasia/intra-epithelial neoplasia that is critical because it predicts the risk of both malignant transformation and metachronous gastric cancer [2].

On the basis of a consensus nomenclature, the so-called Vienna nomenclature, proposed in 1999 [5] and subsequently updated in 2003 [6] for the improvement in endoscopic techniques and their management implications, recently the World Health Organization (WHO) reiterated the classification of "dysplasia" and "intra-epithelial neoplasia" (IEN), using these terms as synonymous. The three following categories should, thus, be considered:

1. *Negative for intra-epithelial neoplasia/dysplasia*
   This category includes benign mucosal processes that are inflammatory, metaplastic or reactive in nature.
2. *Indefinite for intra-epithelial neoplasia/dysplasia*
   Although this term does not represent a final diagnosis, it is commonly used to indicate an ambiguous morphological pattern, especially in doubtful cases on the nature of a lesion, if neoplastic or not, particularly in small biopsies exhibiting inflammation. Taking into account the interpretative problems, it is not uncommon that regenerative changes could be misleading for intra-epithelial neoplasia/dysplasia, particularly in reactive gastritis and at the edge of a benign ulcer or in the postoperative stomach. Therefore, in those cases where inflammation raises the suspicion of an atypical regenerative process, the diagnosis may be clarified by cutting at deeper levels the tissue block, obtaining additional biopsies or after removing possible sources of cellular proliferative alterations.

   Epithelial proliferation may have the characteristics of indefinite dysplasia, when shows

irregular and tubular structures with mucus depletion, high nuclear-cytoplasmic ratio and loss of cellular polarity. Mitotic activity may be brisk mainly near the proliferative zone in the mucous neck region. The glands are usually closely packed and lined by cells with large, hyperchromatic nuclei. An increasing gradient of alterations is appreciated from the base of the glands to their superficial portion.

3. *Intra-epithelial neoplasia/dysplasia*
   This category belongs to epithelial atypical/neoplastic proliferations characterized by variable cellular and architectural atypia but lacking clear evidence of invasive growth. They can have flat, polypoid or slightly depressed growth patterns.

Histologically, they can be distinguished into:

- *Low-grade intra-epithelial neoplasia/dysplasia*
  These lesions are characterized by a modified mucosal architecture, with distorted tubular structures, papillary formation, crypt lengthening with serration and cystic changes. Glands show various degree of mucin depletion.
  Nuclei are usually pseudostratified in the proliferation zone at the superficial portion of the dysplastic tubules.
- *High-grade intra-epithelial neoplasia/dysplasia*
  Important increasing of architectural distortion and prominent cellular atypia are seen in tubules with frequent irregular branching and folding; there is no stromal invasion. Mucin secretion is absent. Nuclei are often cigar-shaped with prominent nucleoli. Increased proliferative activity is present throughout the epithelium.
- *Intramucosal invasive neoplasia/intramucosal carcinoma*
  Carcinoma is diagnosed when the tumour invades into the lamina propria (intramucosal carcinoma).
  To distinguish intramucosal carcinoma from intra-epithelial neoplasia/dysplasia, absence or minimal desmoplasia accompanied by distinct structural anomalies, such as marked glandular crowding, excessive branching and budding, must be observed. Cells of intramucosal invasive neoplasia are usually cuboidal with a high nuclear-cytoplasmic ratio, round nuclei with prominent nucleoli and commonly show loss of cellular polarity.

Although variable, the diagnosis of gastric intra-epithelial neoplasia/dysplasia offers a relevant instrument to gastroenterologists into predicting an incremental risk of progression of these lesions to gastric cancer. If the progression from low-grade dysplasia to adenocarcinoma has been reported to 0–23% of cases within a mean interval from 10 months to 4 years, the rate of malignant transformation increases to 60–85% of cases within a median interval of 4–48 months for high-grade lesions [7–13]. Therefore, on the basis of the different malignant transformation rates of low-/high-grade dysplasia, patients will be treated with the appropriate therapy and included in a proper surveillance programme.

Benign Epithelial Tumours

Benign Gastric Epithelial Polyps

Gastric epithelial polyps are defined as lesions, which lay above the plain of the mucosal surface. The most common polyps are represented by fundic gland polyps that account for up to 77% of all gastric polyps, followed by hyperplastic polyps and adenomas [14, 15].

Fundic gland polyps (FGPs) occur in two different clinical settings: sporadic and syndromic. Development of dysplasia is extremely rare (<1%) in sporadic FGPs, and no association has been reported for progression in gastric cancer [16]. They also affect patients treated with long-term proton pump inhibitors [17, 18]. As sporadic manifestation of familial adenomatous polyposis (FAP) syndrome, numerous FGPs may be present in young patients. Contrary to sporadic FGPs, up to 48% of syndromic FGPs evolves into dysplasia, although progression to carcinoma remains rare [19, 20].

Recently, gastric adenocarcinoma and proximal polyposis syndrome has been identified as a new hereditary autosomal-dominant gastric cancer syndrome: it is characterized by the development of numerous FGPs and is associated with a significant risk of gastric carcinoma [21].

Hyperplastic polyps are often associated with chronic gastritis and *H. pylori* infection. Dysplasia may be found in 1–3% of hyperplastic polyps and is usually associated with lesions larger than 20 mm in diameter and prevalent in individuals over 50 years of age. Complete excision with entire histologic examination of large hyperplastic polyps is believed to be curative even if dysplasia or intramucosal carcinoma is well-documented [22].

Besides the conventional distinction between benign and malignant tumours, because most gastric tumours are epithelial in origin, they are also divided into two major categories [23]:

1. Exocrine, which comprises adenomas and adenocarcinomas
2. (N)endocrine, including carcinoid tumours and (N)endocrine cell carcinomas (NECC)

Gastric Adenomas

Gastric adenomas are characterized by lesions with raised polyps that by definition exhibit low- or high-grade epithelial dysplasia and comprise 0.5–3.75% of all gastric polyps in the Western Hemisphere, in contrast to 9–20% in areas of high-risk gastric cancer [14].

The risk of carcinoma progression of gastric adenomas is related to the size of the lesions and is increased in lesions larger than 2 cm in diameter.

Histologically, gastric adenomas may be classified as tubular, tubulovillous or villous based on the architecture. Gastric adenomas may be also subtyped, based on the epithelial phenotype, into intestinal and gastric types. The intestinal type of adenoma (adenomatous, type I) is more common and contains absorptive, goblet and Paneth cells [24, 25]. It is similar to colonic adenomas with crowded, tubular glands, lined by atypical columnar cells with overlapping, pencillate, hyperchromatic and/or pleomorphic nuclei with pseudostratification and inconspicuous nucleoli, mucin depletion and lack of surface maturation [26].

Gastric phenotype (foveolar, type II) contains cuboidal or low columnar cells, with clear or eosinophilic cytoplasm and showing round-to-oval nuclei [26].

Adenomatous and foveolar types can be immunohistochemically distinguished, since the first type expresses MUC2, CDX2 and CD10, whereas the second one expresses MUC5AC, lacks CD10 expression and exhibits low level of CDX2 [24, 27, 28].

Interestingly, other types of adenoma have been described:

- Pyloric adenomatous lesions in the body/fundus of the stomach of elderly patients, commonly associated with autoimmune gastritis. These lesions are characterized by eosinophilic cuboidal cells with finely granular cytoplasm with round nuclei and limited mitotic activity.
- Paneth cell adenoma, a rare variant composed exclusively of Paneth cells [29, 30].
- Oxyntic gland polyp or adenoma, which likely represents a morphological link to the previously described variant of gastric adenocarcinoma with chief cell differentiation [31].

The clinical and pathological characteristics of gastric adenomas are presented in Table 4.1.

Malignant Epithelial Tumours

Adenocarcinomas

Most gastric malignancies (95%) originate from glandular epithelium and are classified as adenocarcinoma. A multiplicity of environmental and genetic factors influences the aetiologies of this heterogeneous group of tumours, characterized by different morphologies, molecular backgrounds and histogenesis.

Epidemiology

Gastric carcinoma is the fifth most common malignancy worldwide and remains the third cause of death of all malignancies worldwide [32]. Since the disease remains asymptomatic until reaching the advanced stage, 5-year survival rate is relatively good only in Japan, where it

Table 4.1 Clinicopathologic characteristics of gastric adenomas according to histologic subtypes

Adenoma type	Location	Histological features	Association	Malignant transformation
Intestinal (Type I)	Antrum	Elongated hyperchromatic nuclei, focal globet cells and Paneth cells	Gastritis and IM	High
Foveolar (Type II)	Body	Round to oval nuclei, pale or clear cytoplasm, apical mucin	FAP	Controversial
Pyloric	Body	Round bland or atypical nuclei, ground glass cytoplasm	Autoimmune gastritis and IM	High
Paneth cell adenoma		Paneth cells	Gastritis and IM	Controversial
Oxyntic	Fundus/ cardia	Chief cells, mucous neck cells	Some with mild chronic gastritis	None

Modified from Ref. [39]
Abbreviations: *FAP* Familial Adenomatous Polyposis, *IM* intestinal metaplasia

reaches 90% [33], mainly due to early detection, while in European countries, survival rates vary from ~10% to 30% [34]. The incidence shows wide geographical variation: the distribution shifts from areas at high incidence (>60 per 100 000 males), such as in Eastern Asia, Eastern Europe and Latin America, to zones at low incidence (<15 per 100,000 population) as in North America, Northern Europe and most countries in Africa and in Southeast Asia [35]. A predominance of the cancers of antrum and pylorus occurs in high-risk areas, while proximal stomach and oesophagogastric junction adenocarcinomas are relatively more common in low-risk areas [36].

Nevertheless, a general declining incidence of gastric cancer has been observed worldwide in the last few decades, probably also due to the higher standards of hygiene, improvement in food conservation, a high intake of fresh fruits and vegetables and *Helicobacter pylori* (*H. pylori*) eradication [37].

Aetiology and Pathogenesis

The Correa cascade of gastric carcinogenesis [1] shows the progressive changes in the gastric mucosa with metamorphosis of normal gastric mucosa into carcinoma through the subsequent development of inflammation, atrophy, metaplasia and dysplasia [38], involving several genetic alterations (Fig. 4.1).

Several precancerous conditions have been reported, including atrophic gastritis and intestinal metaplasia due to *H. pylori* infection or autoimmunity, gastric ulcers, gastric polyps, previous gastric surgery and Ménétrier's disease. Moreover, associations with environmental agents such as dietary constituents and the generation of carcinogenic N-nitroso compounds within the stomach, in addition to inherited disposition, have also been recognized.

Chronic Gastritis and Intestinal Metaplasia

Chronic gastritis is the most important and well-studied risk factor for the intestinal type of gastric cancer [39]. Even though *H. pylori*-associated and autoimmune gastritis are two different etiologic agents of chronic inflammation, they result into atrophic gastritis that has been shown to precede the development of malignancy [40, 41].

For routine histopathological evaluation, the Sydney Classification System (later updated in Houston) was developed to provide information on the grade, topography (antrum, corpus, incisura) and origin of chronic gastritis [42].

To characterize the degree of chronicity, an international group of gastroenterologists and pathologists (Operative Link on Gastritis Assessment [OLGA]) developed a system for reporting the stage of gastritis, termed the OLGA Staging System [43]. The stage of gastritis is obtained by combining the extent of atrophy as scored histologically with the sites of atrophy identified by multiple biopsies from the antrum, incisura angularis (junctional area between the anatomic antrum and body along the lesser cur-

vature) and corpus according to the Sydney System protocol [42, 43]. A long-term follow-up study reveals that the combination of OLGA Staging System with *H. pylori* status provides relevant information to stratify and confidently manage patients in accordance with their cancer risk [44].

Intestinal metaplasia (IM) is characterized by wide heterogeneity. In this regard, three types of IM have been recognized:

- Type I (complete or small intestinal type) consists of mature enterocytes with brush borders, Paneth cells and goblet cells, the latter secreting sialomucins [45]. The genetic characterization of complete-type IM evidences the expression of intestinal mucin MUC2 and markedly decreased levels of gastric mucins MUC1, MUC5AC and MUC6 [46]. Among IM subtypes, type I has been reported to be the predominant (73%) in biopsies, and it has been seen as the most common in benign conditions, 70% in gastric ulcers and 76% in chronic gastritis [47].
- Type II IM (incomplete, immature or colonic type) is characterized by few or absent absorptive cells and the presence of columnar "intermediate" cells in various stages of differentiation, secreting neutral and acid sialomucins, and goblet cells secreting sialomucin or occasionally sulphomucins [45, 47]. Differently to type I, in incomplete-type IM, gastric mucins are co-expressed with intestinal mucin MUC2. These expression patterns indicate that incomplete-type IM is a phenotypic mixture of gastric and intestinal cells, reflecting differentiation anomalies [46].
- Type III IM, in which the predominant mucin secreted by the "intermediate" cells is acid sulphomucin rather than sialomucin as in type II IM [47, 48]. Both type II and III incomplete IMs maintain the expression of gastric mucins MUC1, MUC5AC and MUC6 [46]. At molecular level, all types of IM express the intestinal transcription factor CDX2, generally expressed in the normal bowel [49]. Type III IM, which has been identified in only 9.8% of all biopsies with IM, has a higher incidence in carcinoma (35%) than in benign conditions (7%) [47].

Albeit some studies have demonstrated that cancer risk is increased from type I to type III of IM [47, 48, 50, 51], currently, subtyping of IM is not recommended in routine practice, because there is no conclusive evidences of the association between these subtypes and the risk of gastric cancer [39].

Recently, OLGA system has been modified for the assessment of IM (OLGIM) for the staging of chronic gastritis [52]. With respect to OLGA system, essentially based on atrophy, OLGIM system provided a significantly higher agreement between pathologists. However, the practical value of this system in predicting the development of dysplasia or cancer needs to be addressed. At present, the reversibility of metaplasia involving the gastric mucosa is considered controversial. Although eradication of *H. pylori* has been associated with the reversibility of IM in some studies [53–55], in other studies cancer risk decreased only after eradication in patients with nonatrophic mucosa [56–58].

Another pattern of metaplasia, spasmolytic polypeptide-expressing metaplasia (SPEM), is a metaplastic mucous cell lineage with morphological features and the phenotype of deep antral glands, including strong expression of trefoil factor (TFF)2, a member of small secretory peptides, which plays a role in the protection and repair of the gastrointestinal mucosa [59], and MUC6.

It has been reported that SPEM is strongly associated with *H. pylori* infection, since it was detected in 68% of infected patients, and it has been also seen in the setting of autoimmune atrophic gastritis targeting parietal cells in the corpus [60]. Moreover, recent studies have shown that SPEM is associated with 90% of gastric adenocarcinomas and have suggested that SPEM may play a role in the preneoplastic process [60–62].

Helicobacter pylori Infection and Gastric Cancer

H. pylori is the most common chronic pathogen in humans, since more than 50% of the world

population is infected. Nowadays, it is the only bacteria classified as a class I carcinogen by the WHO [63], as confirmed by numerous epidemiologic studies on the association of *H. pylori* infection and risk of gastric cancer. However, considering that only 1–3% of infected people actually develop gastric cancer, it has been suggested that other factors, including the host, may also play a role in carcinoma development [64, 65].

On the basis of the undoubted strong correlation between *H. pylori* infection and gastric cancer, the Maastricht III Guidelines recommend to treat the infection in peptic ulcer diseases, mucosa-associated lymphoid tissue lymphomas, atrophic gastritis, patients after resection of gastric cancer, first-degree relatives of gastric cancer patients, patients with unexplained iron deficiency anaemia, patients with idiopathic thrombocytopenia purpura, patients who require long-term nonsteroidal anti-inflammatory drugs (NSAIDs) and patients who just wish to be treated [66].

Diet

Diet exerts an important role in gastric carcinogenesis, especially in intestinal-type adenocarcinoma and in combination with *H. pylori* infection [67–69]. In this regard, high intake of fresh fruits and vegetables, Mediterranean diet, a low-sodium diet, salt-preserved food, red and high cured meat, adequate alcohol intake and maintaining a proper body weight might be associated with a decreased risk of gastric cancer [70–72]. Functional foods such as fresh fruits and dark green, light green and yellow vegetables rich in β-carotene, vitamins C and E and folate may exhibit a protective action in gastric cancer, probably due to their antioxidant effect. Among these compounds, β-carotene seems to be the leading risk reducer [73]. Nevertheless, the outcomes of a recent meta-analysis of randomized trials comparing the effect of antioxidant supplements with placebo or no intervention did not show a significant effect on the incidence of gastric cancer [74], even though the nutritional basic conditions of the populations seem to influence the results [75]. On the contrary, high plasma concentration of carotenoids, α-tocopherol and retinol was found to be associated with reduced risk of gastric cancer [76]. Therefore, further investigations are required.

Smoking

Several studies have confirmed that tobacco smoking increases the risk of gastric cancer, both cardia and non-cardia subtypes [77, 78]. The risk of gastric carcinoma is increased by 60% in male and 20% in female smokers compared to non-smokers. Moreover, this risk decreases in former smokers compared with occasional smokers, while smokers with higher consumption of cigarettes (>20 cigarettes per day) have a higher risk to develop gastric cancer [77].

The Operated Stomach and Cancer

Gastric stump cancer is a carcinoma that occurs in the gastric remnant at least 5 years after the surgery for peptic ulcer [79]. This gastric cancer subtype represents from 1.1% to 7% of all gastric carcinomas, with a prevalent disposition in male [80–82]. Gastrectomy is a well-documented risk factor for gastric stump cancer, even long time after the initial surgery [83, 84]: in fact, after 15 years from the gastrectomy, the risk to develop this cancer is increased from four- to sevenfold if compared with the general population [83, 85].

The infection with Epstein-Barr virus (EBV), a human herpesvirus for which a causal role in gastric carcinogenesis has been suggested [86], is more often present in gastric remnants than in intact stomachs [87] and may interact with the p53 protein [88]. In contrast, *H. pylori* infection in gastric stump cancer is less frequent [89]. Well-defined precursor lesions, mostly by dysplasia, commonly precede gastric stump cancer and therefore, endoscopic surveillance with multiple biopsies of the gastroenterostoma is recommended [90].

Pathology

Several systems have been proposed to classify gastric adenocarcinoma on the basis of macroscopic features (Borrmann) [91] or exclusively on the histological tumour growth pattern (Ming, Carneiro, Goseki) [92–94]. The two most commonly used histological classifications are the

Lauren and World Health Organization (WHO) systems (Table 4.2) [3, 95]. More recently, molecular classifications based on gene expression profiles and proteomics have been proposed; however, these have not yet used in routine [96–98].

Topography and Macroscopic Features of Gastric Adenocarcinoma

Adenocarcinoma may occur everywhere in the gastric mucosa. From the classification point of view, it is important to distinguish the oesophagogastric junction (OGJ) cancer from any other site in the gastric wall. The term oesophagogastric junction (OGJ) corresponds to the anatomical region where the oesophagus ends and the stomach begins. Several classification systems of OGJ tumours have been proposed on the basis of localization of tumour epicentre with respect of OGJ.

According to WHO classification:

1. Adenocarcinomas crossing the OGJ are considered as adenocarcinoma of OGJ, without taking into account the localization of remaining bulk of tumour.
2. Adenocarcinomas located entirely above the OGJ are considered to be oesophageal carcinomas.
3. Adenocarcinomas located entirely below the OGJ are considered as gastric carcinomas, also referred to as "adenocarcinoma of proximal stomach".

Table 4.2 Laurén and World Health Organization classification systems of gastric cancer

Laurén	World Health Organization 2010
Intestinal type	Papillary adenocarcinoma Tubular adenocarcinoma Mucinous adenocarcinoma
Diffuse type	Poorly cohesive carcinoma (including signet-ring cell carcinoma and other variants)
Mixed type	Adenocarcinoma and undifferentiated carcinoma
Indeterminate	Adenosquamous carcinoma Carcinoma with lymphoid stroma (medullary carcinoma) Hepatoid adenocarcinoma Squamouscell carcinoma

Modified from Ref. [39]

For the latter one, in 2017, the 8th Edition of Union for International Cancer Control (UICC) TNM classification [99] has proposed some modifications in the staging assessment, based on the tumour epicentre and tumour extension. Similarly, the TNM classification of the American Joint Committee on Cancer (AJCC) [100] refers to gastric or oesophageal staging systems, with a slight difference in the definition of anatomical limit of location of tumour epicentre (see also section "Staging of Gastric Carcinoma" in this chapter).

Advanced gastric carcinoma can display various macroscopic features. As previously mentioned, Borrmann's classification is the most commonly used. This classification divides gastric carcinoma into four distinct types [91] as reported in the Fig. 4.2.

Polypoid and fungating tumours typically consist of friable, ulcerated masses that bleed easily and project from a broad base in the gastric lumen. Characterized by sharp raised margins, they tend to develop in the body of stomach, in the region of greater curvature, posterior wall or fundus (see Fig. 4.2a, b).

Ulcerated carcinomas occur frequently in the OGJ, antrum or lesser curvature. They can be distinguished by benign ulcers for an irregular margin with raised borders and thickened, uneven and indurated surrounding mucosa. Furthermore, malignant ulcers tend to be larger than the benign ones. Nevertheless, in many malignant ulcers, these typical features are absent; thus endoscopic appearance should be supported by complemented biopsies (Fig. 4.2c).

Invasive adenocarcinoma may spread superficially in the mucosa and submucosa or infiltrates the wall (see Fig. 4.2d) which may become diffusely indurated as a consequence of an intense desmoplastic reaction (linitis plastica, Fig. 4.3). In such cases, there is usually non-visible localized growth.

Outer of classified types, other gastric carcinomas can secrete a considerable amount of mucins, which confers to tumours a gelatinous appearance at naked eye, such to be defined as mucinous or colloid carcinomas.

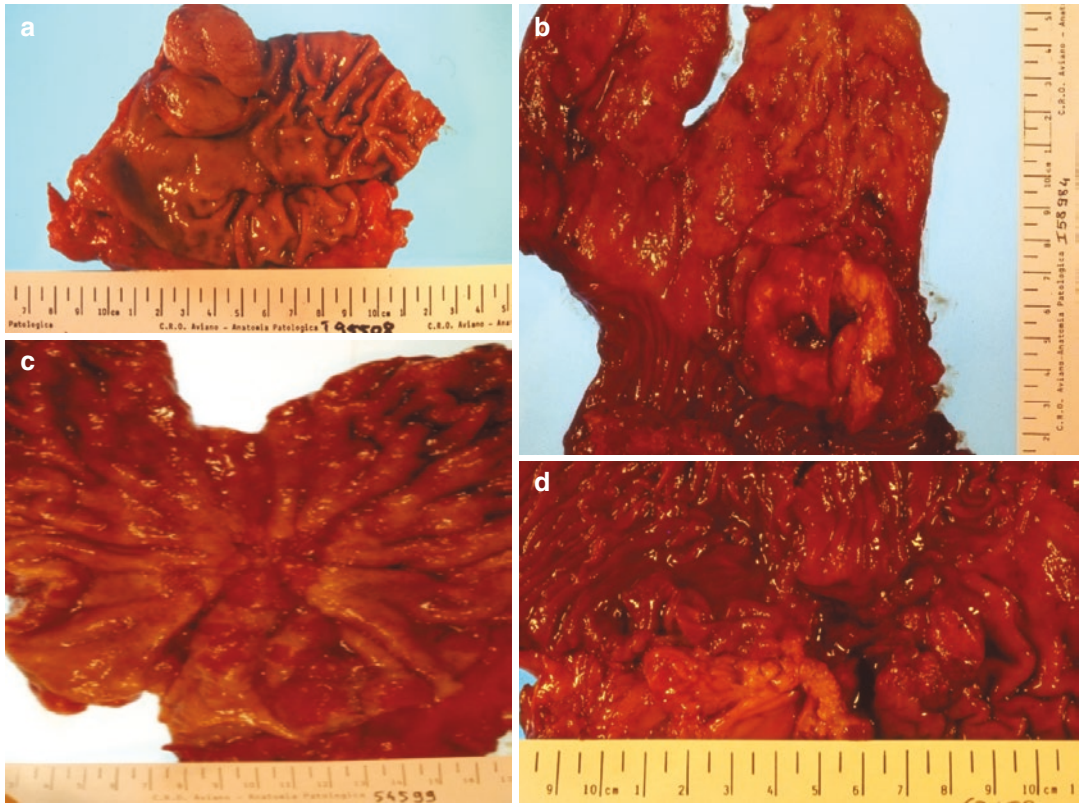

Fig. 4.2 Borrmann classification of gastric carcinomas. (**a**) type I, polypoid; (**b**) type II fungating, (**c**) type III ulcerated; (**d**) type IV, scirrhous, diffusely infiltrating

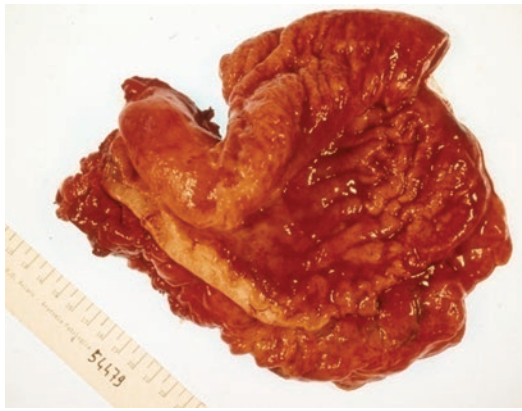

Fig. 4.3 Linitis plastic

Microscopic Features

At microscopic level, proximal and distal gastric adenocarcinomas show similar characteristics, despite their epidemiological differences. On the basis of contributions emerging from intratumoural variations in architecture and/or differentiation, several histological classifications have been proposed over the years.

The WHO Classification

In 2010, the WHO revised the classification of gastric adenocarcinoma, without taking into account the histogenesis and differentiation, but according to the morphological patterns commonly exhibited by tumours in other gastrointestinal sites, such as the small bowel, ampulla of Vater and colon [3]. The 2010 WHO classification recognizes five major types of gastric adenocarcinoma based on the predominant histological growth pattern: (1) papillary, (2) tubular, (3) mucinous (tumours with mucinous pools exceeding 50% of the tumour), (4) poorly cohesive (including signet-ring cell carcinoma and other variants) and (5) mixed adenocarcinomas

(Table 4.2) [3]. Uncommon variants of gastric carcinomas include the squamous cell, adenosquamous, hepatoid, parietal cell, Paneth cell, micropapillary, undifferentiated subtypes and carcinoma with lymphoid stroma (medullary carcinoma) [3, 101].

Tubular Adenocarcinomas

Branching tubules, varying in their diameter and acinar structures, are the main morphological features. Individual tumour cells are columnar, cuboidal or flattened by intraluminal mucin. The degree of cytological atypia varies from low- to high-grade. Tubular adenocarcinoma may show morphological variants, from a poorly differentiated, sometimes called solid carcinoma, to a lymphoid stroma-rich tumour (medullary carcinoma) or a desmoplastic tumour.

Papillary Adenocarcinomas

Papillary adenocarcinomas are well-differentiated exophytic carcinomas with elongated frond-like projections lined by cylindrical or cuboidal cells, with fibrovascular connective tissue cores. Some tumours show tubular differentiation (tubulopapillary). The degree of cellular atypia and mitotic index may be variable.

Mucinous Adenocarcinomas

Extracellular mucinous pools must represent 50% or more of the tumour. Glands are lined by a columnar mucous-secreting epithelium, together with interstitial mucin, or the tumour is composed by chains or irregular cell clusters floating in mucinous lakes. A discrete component of signet-ring cells may be present but usually is not prominent.

Poorly Cohesive Carcinoma, Including Signet-Ring Cell Carcinomas

More than 50% of the tumour consists of isolated or small groups of malignant cells containing intracytoplasmic mucin that frequently displaces the nuclei at the periphery of the cytoplasm, creating a classical signet-ring cell appearance due to a globoid, optically clear cytoplasm. Other diffuse carcinomas contain cells with central nuclei resembling histiocytes, showing little or no mitotic activity; or small, deeply eosinophilic cells with or without mucins or finally anaplastic cells devoid of mucin.

These cell types intermingle with one another and constitute varying tumour proportions. Typically, signet-ring cell carcinomas may harbour diffuse desmoplasia with dispersed tumour cells in the stroma. Cytokeratin immunostaining may be useful in some difficult case in order to establish the diagnosis and the extent of disease in the gastric wall.

Mixed Carcinomas

These gastric carcinomas are composed of a mixture of morphologically identifiable glandular (tubular/papillary) and poorly cohesive cellular histological components. Mixed carcinomas have been shown to be clonal [102, 103] with phenotypic divergence attributed to somatic mutation in E-cadherin gene (CDH1) and restricted to the poorly cohesive component [104]. Epigenetic changes have also been seen to be implicated in the histogenesis of mixed carcinoma [105].

The Laurén Classification

The Laurén classification is applied in routine practice by pathologists, and it is commonly used by epidemiologists and clinicians for evaluating the natural history of gastric adenocarcinoma, especially with regard to incidence trends and etiologic precursors [106], although all existing classifications of gastric adenocarcinoma, including Laurén's one, are of limited significance in terms of therapeutic decisions [97].

In this classification system, the tumours are distinct in two types: intestinal or diffuse. Tumours which present an equal proportion of intestinal and diffuse components are referred to as mixed carcinomas. At the same way, tumour cells that are too undifferentiated to be categorized in the reported types are assigned in the indeterminate category.

Intestinal Carcinomas

As the most common subtype, the intestinal carcinoma occurs in about 54% of the cases, with a prevalence twofold higher in males compared to females and localized mostly in the antrum. Histopathologically, it is characterized by recognizable glands that range from well differentiated

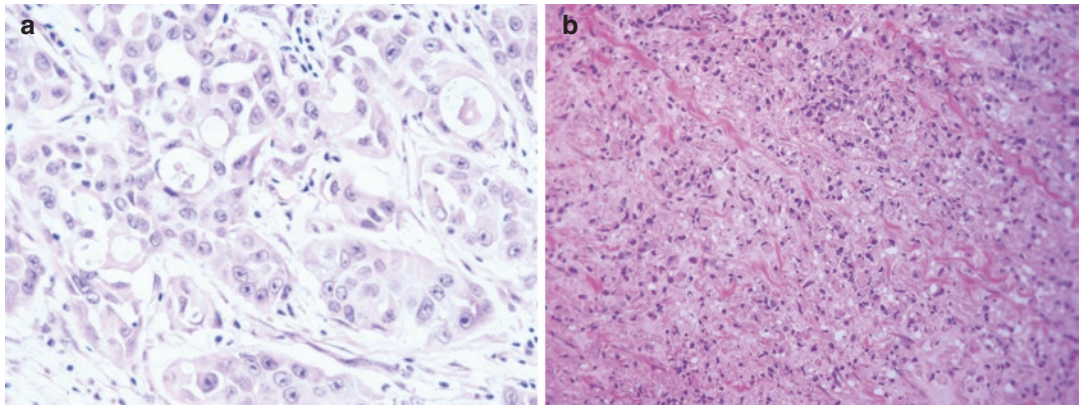

Fig. 4.4 Laurén classification of gastric adenocarcinomas. (**a**) Intestinal type; (**b**) diffuse type

to moderately differentiated, sometimes with poorly differentiated tumour areas (Fig. 4.4a). Intestinal carcinomas typically arise on a background of intestinal metaplasia. However, at cellular level, despite their classification into intestinal carcinomas, these cells may show morphological and immunological differentiation typical of gastric and gastrointestinal cells, or null differentiation, over the intestinal type. For this reason, the prognostic relevance of Laurén classification remains controversial [107].

Diffuse Carcinomas

By contrast, the diffuse subtype (32%) is characterized by tumour cells that are poorly cohesive and diffusely infiltrating the gastric wall with little or no gland formation (Fig. 4.4b). Small amounts of interstitial mucin may be present. Desmoplasia is more pronounced and associated inflammation is less evident in diffuse cancers than in the intestinal carcinomas.

This subtype occurs equally often in males and females, and these patients are on average younger than those with intestinal carcinomas. The intestinal type of gastric cancer is felt to be caused mainly by environmental (exogenous) factors, whereas the diffuse type is thought to be due to hereditary and genetic (endogenous) factors [108].

Albeit the intestinal and diffuse gastric carcinoma subtypes are pathologically considered as separate entities, from the clinical point of view, they are treated similarly. Clinically, the main difference is related to the distinct recurrence patterns, with the diffuse-mixed types more prone to peritoneal dissemination, especially when the serosa is involved, whereas the risk of liver metastases is higher in the intestinal type [108].

Comparing the Laurén and the WHO classifications, tubular and papillary adenocarcinomas fall within the intestinal type of stomach cancer, whereas signet-ring cell carcinoma and other poorly cohesive carcinomas correspond to the Laurén diffuse type (Table 4.2) [109].

Goseki Classification

The Goseki classification divides gastric cancer, based on intracellular mucin production and the degree of tubular differentiation, into four groups:

1. Group I: tubules well differentiated, intracellular mucin poor
2. Group II: tubules well differentiated, intracellular mucin rich
3. Group III: tubules poorly differentiated, intracellular mucin poor
4. Group IV: tubules poorly differentiated, intracellular mucin rich

Notably, prognostic value has been attributed to this classification system [94, 110].

The Dawn of Phenotypic Classification

A classification based on four histotypes has been proposed by Carneiro and colleagues in 1997 [111]:

1. Glandular and isolated cell carcinomas (approximately equivalent to the intestinal and diffuse carcinomas of Laurén classification)
2. A solid variety (composed of sheets, trabeculae or islands of undifferentiated cells with no glandular formation)
3. A mixed type consisting of glandular and isolated cell types mixture

This classification has been shown to have a prognostic significance [112].

The introduction of markers of cell differentiation allowed to obtain more information on the tumours histogenesis and classification. The following markers have been used:

- Mucin MUC5AC and trefoil peptide TFF1 as markers of surface gastric epithelium (foveolar cells) (Fig. 4.5a)
- MUC6 and TFF2 as markers of mucous neck cell, pyloric gland and Brunner's gland cells
- MUC2, CDX2 and CD10 as intestinal cell markers (Fig. 4.5b)

As a consequence, four phenotypes of gastric carcinomas have been identified:

1. Gastric
2. Mixed gastric and intestinal (further divisible in predominant gastric or intestinal type)
3. Intestinal
4. Unclassifiable or null phenotype (lack of these markers) [113–115]

Additionally, pepsinogen-1 staining helped to distinguish mucous neck/pseudo-pyloric type from true pyloric type [115].

This classification has evidenced the limit of Laurén classification into identifying tumours showing gastric phenotype (positive for selected markers) with the inappropriate term of "intestinal" carcinomas. Therefore, these findings had consequences also on the classical multistep process of gastric carcinogenesis [111, 116–118].

Although current histopathological systems influence endoscopic or surgical choices, they are still insufficient to guide precision treatments for individual patients. Not only new therapies, but a new classification for gastric carcinoma is needed as well [108].

The overwhelming majority of common gastric cancers are adenocarcinomas, for which the origin from a progenitor cell specializing towards an exocrine cell lineage has been hypothesized [119]. Nevertheless, several reports have shown that (neuro)endocrine markers chromogranin A (CgA) and/or synaptophysin (Syn) have been found immunohistochemically in about 15–70% of conventional gastric adenocarcinomas, on the basis of different criteria applied or variation in the sensitivity of antibodies used [120–124].

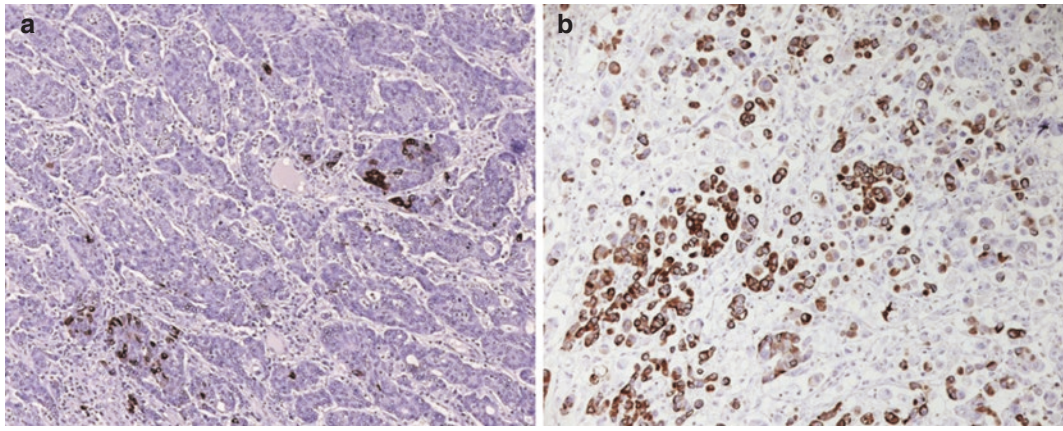

Fig. 4.5 Expression of (**a**) gastric mucin MUC5AC and (**b**) intestinal mucin MUC2 in two different cases of gastric adenocarcinoma

Jiang et al. [125] reported that adenocarcinomas of the stomach with more than 20% of the tumour cells expressing CgA and/or Syn, the so-called large cell neuroendocrine carcinomas (LCNEC), significantly correlated with a poorer overall survival rate than adenocarcinomas without endocrine differentiation or up to 20% of tumour cells expressing CgA and/or Syn (adenocarcinomas with neuroendocrine differentiation, ACNED).

Canzonieri et al. [23] evaluated the diagnostic and prognostic implications of endocrine differentiation in 103 common gastric adenocarcinomas (n = 71) and undifferentiated carcinomas (n = 32). Maturely differentiated exocrine and endocrine phenotypes were evaluated by using gastric exocrine markers (MUC5AC and MUC6) and endocrine markers (gastrin and somatostatin, in CgA- and/or Syn-positive tumours) along with intestinal exocrine (MUC2, villin and CD10) and endocrine markers (glucagon-like peptide-1, GLP-1, and gastric inhibitory polypeptide, GIP, in CgA- and/or Syn-positive tumours).

Immunohistochemical analysis revealed that 66 tumours (64%) were positive for generic endocrine markers such as CgA and/or Syn. The 14 patients with more than 20% tumour cells positive for at least 1 endocrine marker (LCNEC) experienced a poorer prognosis than patients with no (n = 37) or 1% to 20% (n = 52) positivity (ACNED). The 16 carcinomas expressing the maturely differentiated exocrine gastric phenotype significantly correlated with poorer outcome compared with carcinomas with mature exocrine intestinal (n = 22) or mixed/gastrointestinal phenotypes (at least 1 gastric and 1 intestinal exocrine phenotype marker simultaneously positive, n = 64).

Among tumours expressing CgA and/or Syn, the maturely differentiated endocrine gastric phenotype (n = 26) was a negative prognostic factor compared with mature endocrine intestinal (n = 21) and mixed/gastrointestinal (n = 5) phenotypes.

On the basis of these results, it has been demonstrated that endocrine differentiation and maturely exocrine/endocrine gastric phenotypes are associated with an unfavourable prognosis and may identify subsets of patients for tailored therapy [23].

Unusual Variants of Gastric Carcinoma

Several other uncommon histological variants of gastric carcinomas exist (5%) that are not an integral part of the above-mentioned classification system.

Adenosquamous and Squamous Cell Carcinoma

To be diagnosed, neoplastic squamous cells, characterized by keratin pearl formation and intercellular bridge, in addition to glandular element, must be detected in primary adenosquamous carcinoma [126]. At ultrastructural level, these neoplastic cells showed differentiation features typical of both squamous and adenosquamous cells, supporting the hypothesis of their origin from multipotent stem cells [127]. These tumours are often localized in the antrum and show lymphovascular permeation. This variant may pose some problems of interpretation of peculiar findings:

1. Metastases usually contain both glandular and squamous components, but, sometimes, only one component may be present.
2. A tumour with a distinct boundary between the two components may represent a collision tumour.
3. Tumours containing discrete foci of benign-appearing squamous metaplasia are termed adenocarcinomas with squamous differentiation (adenoacanthoma).

Pure squamous cell carcinomas develop rarely in the stomach [128, 129] and are usually diagnosed in advanced stages, thus having a poor prognosis [130]. Pure squamous cell carcinoma of the stomach possibly arises from squamous metaplasia of an adenocarcinoma, from heterotopic squamous epithelium or from multipotent stem cells showing bidirectional differentiation [130, 131].

Hepatoid Adenocarcinoma

Large polygonal eosinophilic hepatocyte-like neoplastic cells can be observed as interspersed elements in a small number of carcinomas of the stomach. These neoplastic cells may produce consistent amount of α-fetoprotein (AFP), as revealed by in situ detection and in the serum [132]. Hepatoid adenocarcinoma has generally been reported in patients older than 50 years, even though it has been occasionally diagnosed in younger patients [133, 134]. These carcinomas are characterized by advanced bulky polypoid tumours with ulceration, necrotic and haemorrhagic areas. Antrum is the most common site where the development of hepatoid adenocarcinoma has been described, followed by the fundus and, with a lower frequency, the cardia [133]. Tumour heterogeneity is demonstrated by hepatoid foci mixed with adenocarcinoma, often presenting papillary pattern, and less differentiated areas characterized by giant and spindle cells [132–136].

Since these cells express typical markers of intestinal cells, the histogenesis of hepatoid adenocarcinoma from an intestinal phenotype has been suggested [137]. Clinical evidences showed an extensive vascular infiltration of these adenocarcinomas, as demonstrated by the high incidence of liver and lymph node metastases and poorer prognosis compared with typical adenocarcinoma of the stomach [132, 134, 138, 139]. At molecular level, the presence of albumin, AFP, α_1-antichymotripsin and bile production have been demonstrated by immunohistochemical and in situ hybridization studies [140–142]. Recently, PLUNC (palate, lung and nasal epithelium carcinoma-associated protein) has been described as hepatoid adenocarcinoma marker [143].

Since it is difficult to distinguish a liver metastasis from gastric hepatoid adenocarcinoma and primary hepatocellular carcinoma (HCC) in a liver biopsy, it is useful adopting the immunostaining for Hep-Par-1 that extensively stains most HCCs, whereas only focal staining of Hep-Par-1 is observed in gastric hepatoid adenocarcinoma [144].

Gastric Choriocarcinoma

For a pathological diagnosis of choriocarcinoma of the stomach, the assessment of cytotrophoblast and syncytiotrophoblast is a prerequisite, and it can be confirmed by human chorionic gonadotropin (hCG)-positive cells in immunohistochemical tests [145–148] and by high levels of hCG in the blood [146, 147, 149]. Other histological features are intratumoural haemorrhage, necrosis and vascular invasion.

Gastric Carcinoma with Lymphoid Stroma

Infection with Epstein-Barr virus (EBV) has been detected in up to 18% of gastric carcinomas [150], and over 80% of gastric lymphomas are related to EBV infection [151]. These gastric carcinomas, with well-defined margins, prevalently occur at the proximal stomach, including at the stump of patients undergoing subtotal gastrectomy [152]. At histological level, the tumours are typically composed of irregular sheets, trabeculae, ill-defined tubules or syncytia of polygonal cells mixed with a prominent lymphocytic infiltrate.

As revealed by immunophenotypic analysis, the main component of the infiltrate is represented by CD8-positive T cells, followed in a minor extent by B lymphocytes, plasma cells, neutrophils and eosinophils and more rarely by giant cells [153]. The main differential diagnosis, in these cases, is with the gastric lymphomas. Compared to typical gastric carcinomas, gastric lymphomas occur in slightly younger age and prevalently in males [152, 154, 155] and has a better prognosis [87, 154, 156, 157].

Albeit it is not detected in normal gastric mucosa or intestinal metaplasia, EBV is revealed in dysplasia [158]. However, further investigations concerning the role of EBV are required to clarify if this virus intervenes directly in carcinogenesis process or occurs as secondary infection [159] in early stage.

Gastric Carcinosarcoma

Carcinosarcoma of the stomach is a rare biphasic tumour that consists of both carcinomatous and

sarcomatous components. The sarcomatous components may be differentiated into leiomyosarcoma, rhabdomyosarcoma and osteosarcoma [160–163]. Occurrences of adenosquamous components combined with neuroendocrine cells have also been reported in these neoplasms [164–167]. Gastric carcinosarcoma is usually associated with poor prognosis [168].

Micropapillary Carcinoma

Micropapillary carcinoma is rare and histologically is characterized by irregular small clusters of neoplastic cells in clear lacunar spaces simulating lymphatic or vascular channels. Micropapillary carcinomas differ from tubular or papillary carcinomas because, histologically, they lack fibrovascular stalks and show an extensive lymphovascular tumour invasion and high metastatic potential. Moreover, the recognition of a micropapillary carcinoma component, which may range from 5% to 80%, is important, because it is associated with poorer prognosis in an otherwise common adenocarcinoma [101, 169].

Parietal Cell Carcinoma

Bulky lesions at both gastric body and antrum level have been reported as prevalent features of these exceedingly rare tumours [170]. With an expanding growth pattern, these neoplasms present sheets of cells containing small gland-like clefts. These tumour cells are similar to acid-secreting parietal cells, since they have eosinophilic granular cytoplasm and positively stain for PTAH (phosphotungstic acid haematoxylin) and Luxol fast blue. Moreover, they are immunopositive for parietal cell-specific antibodies, for H^+/K^+ ATPase and for human milk fat globule-2. Additionally, ultrastructural evaluation reveals numerous mitochondria and intracellular canaliculi [170–172]. Lymph node metastases are not particularly extensive, and the prognosis seems to be more favourable than other usual gastric adenocarcinomas [171].

Gastric Mucoepidermoid Carcinoma

These neoplasms are extremely rare and show mixed morphological features between mucus-producing and squamous epithelia [173].

Paneth Cell Carcinoma

As indicated by its designation, the predominant cells in these tumours are Paneth cells, which show eosinophilic cytoplasmic granules that are immunopositive for lysozyme [174, 175]. However, it must be noted that Paneth cells can be found dispersed among typical gastric adenocarcinomas [30, 176].

Gastric Malignant Rhabdoid Tumour

With poor prognosis, this carcinoma is characterized by poorly cohesive, round-to-polygonal cells with eosinophilic or clear cytoplasm and large nuclei with predominant nucleoli. In addition to a strong immunoreactivity to vimentin, these cancer cells are also immunopositive for cytokeratin, epithelial membrane antigen (EMA) and focal neuron-specific enolase (NSE) and immunonegative for carcinoembryonic antigen (CEA) [177–179].

Undifferentiated Carcinoma

With this terminology are indicated all gastric tumours that don't exhibit any differentiation, but only epithelial phenotype at least in part (e.g. cytokeratin expression) and that fall into indeterminate category of Laurén classification.

Staging of Gastric Carcinoma

Early Gastric Cancer

Early gastric cancer (EGC) is defined as intramucosal or submucosal tumour invasion independent of lymph node involvement [96]. Although the designation seems to be linked to the stage of cancer genesis, the term EGC indicates the possibility for these neoplasms to be cured [180]. Nevertheless, if not treated, 63% of EGC cases have been seen to evolve in advanced tumours within 5 years [181]. Albeit EGC represents 15–21% of all gastric cancers in the Western world, it accounts for more than 50% of the gastric carcinomas in Japan [3, 182, 183]. It could be speculated that these differences are probably related to endoscopic screening programmes implemented in Japan, although differences in

diagnostic criteria may also play a role [39]. Specifically, most EGCs show dimensions ranging between 20 and 50 mm and occur in the lesser curvature and around the angulus [184, 185]. EGCs may exhibit different behaviours in terms of invasiveness: if some expanded only in the lateral sense, others, although the reduced size (3–5 mm), can invade into the submucosa [186, 187].

On the basis of endoscopic appearance, the Japanese Gastric Cancer Association classified EGCs as protruded (type I), elevated (type II), including the subtypes IIa (elevated type), IIb (flat type) and IIc (depressed type), and excavated (type III).

Histologically, especially for minute EGCs (<20 mm), a well-defined glandular differentiation can be observed, even though histological changes occur in the course of cancer development. Moreover, a weakly correlation of reported types with microscopic appearance could be observed: in fact, over 50% of EGCs corresponds to the tubular variant and 30% to the papillary one, which are, respectively, associated with types I and II of Japanese classification. Similarly, signet-ring cell carcinoma and poorly differentiated carcinoma represent 25% and 15% of EGCs and are usually associated with types IIc and III [182, 184, 188].

The risk of deep and multifocal penetration into the submucosa and the risk of lymphatic invasion are higher in type IIc, the depressed variant of type II.

Advanced Gastric Cancer

A substantial modification of TNM classification of gastric tumours has been operated in 2017 [99, 100].

As it has been reported in Table 4.3, the modification consists in the subdivision of T1 in two entities to distinguish the depth of tumour invasion in the mucosa and submucosa, the transition of stage T2a into T2 (muscularis propria) and T2b into T3 (subserosa), followed by the redefinition of tumour which penetrates the serosa or invades adjacent structures as, respectively, T4a and T4b, rather than as T3 and T4.

Concerning the staging of adenocarcinoma of the OGJ, it depends on the location of epicentre: if the centre of tumour, which extends into the oesophagus, is located within 2 cm of the OGJ, the adenocarcinoma is staged in accordance to the scheme of the oesophageal carcinoma. Alternatively, as it has been proposed by TNM classification of UICC, tumours of which epicentre in the stomach is more than 2 cm far from the OGJ are staged according to the scheme for gastric carcinoma, even if the OGJ is involved [99]. The TNM classification of AJCC is essentially in agreement with these definitions, but in the case of tumours centred within 2 cm of the OGJ, they can be staging by gastric carcinoma system, if they not cross the OGJ [100].

Pattern of Spread

Gastric carcinomas can spread by direct extension to adjacent organs, lymphatic and/or peritoneal dissemination.

Direct Extension of the Tumour

When the serosa was penetrated, gastric cancer cells can spread to the pancreas, liver, spleen, transverse colon and greater omentum. Often, an early transperitoneal dissemination can be observed. It has been reported that neoplasms at the OGJ infiltrate into the wall at the lower end of the oesophagus, whereas tumours at distal level tend to microscopically extend within the duodenum [189]. Carcinomas composed by poorly cohesive cells preferentially metastasize to serosal surface and show a widespread intramural permeation of small lymphovascular vessels. Therefore, these neoplasms commonly invade the duodenum via either submucosal or subserosal routes or the submucosal lymphatics [190]. Duodenal invasion occurs more frequently than expected based on gross examination. Therefore, resection margins should be monitored by intraoperative consultation.

Lymphatic Spread

A deeper invasiveness of tumour correlates with a major incidence of lymph node metastases [191], of which distribution differs according to the tumour location, but commonly involved nodes along the lesser and greater curves of the

Table 4.3 TNM classification of gastric tumours

Carcinoma of the stomach			
T – Primary tumour			
TX	Primary tumour cannot be assessed		
T0	No evidence of primary tumour		
Tis	Carcinoma in situ		
T1	Tumour invades lamina propria, muscularis mucosae or submucosa: T1a: Tumour invades lamina propria or muscularis mucosae T1b: Tumour invades submucosa		
T2	Tumour invades muscularis propria		
T3	Tumour penetrates subserosal tissue without invasion of visceral peritoneum or adjacent structures[a]		
T4	Tumour perforates serosa or adjacent structures T4a: Tumour penetrates serosa (visceral peritoneum)[b] T4b: Tumour directly invades adjacent organs or structures[c]		
N – Regional lymph nodes[d]			
NX	Regional lymph node(s) cannot be assessed		
N0	No regional lymph node metastases		
N1	Metastases in 1-2 regional lymph nodes		
N2	Metastases in 3-6 regional lymph nodes		
N3a	Metastases in 7-15 regional lymph nodes		
N3b	Metastases in 16 or more regional lymph nodes		
	Regional lymph nodes groups		
	Perigastric lymph nodes		
	Perigastric along greater curvature		
	Perigastric along lesser curvature		
	Right and left paracardial (cardio-oesophageal)		
	Suprapyloric		
	Infrapyloric		
	Second tier nodes		
	Left gastric artery		
	Celiac artery		
	Common hepatic artery		
	Hepatoduodenal (along proper hepatic artery, including portal)		
	Splenic artery		
	Splenic hilum		
M – Distant metastasis			
M0	No distant metastases		
M1[e,f]	Distant metastases Liver Peritoneum Non-regional lymph nodes Lung, CNS – less common		
Clinical Stage Groups (cTNM)			
Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage I	T1 T2	N0 N0	M0 M0
Stage IIA	T1 T2	N1, N2 or N3 N1, N2 or N3	M0 M0
Stage IIB	T3 T4a	N0 N0	M0 M0

(continued)

Table 4.3 (continued)

Stage III	T3	N1, N2 or N3	M0
	T4a	N1, N2 or N3	M0
Stage IVA	T4b	Any N	M0
Stage IVB	Any T	Any N	M1
Pathological Stage Groups (pTNM)			
Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T1	N1	M0
	T2	N0	M0
Stage IIA	T1	N2	M0
	T2	N1	M0
	T3	N0	M0
Stage IIB	T1	N3a	M0
	T2	N2	M0
	T3	N1	M0
	T4a	N0	M0
Stage IIIA	T2	N3a	M0
	T3	N2	M0
	T4a	N1	M0
	T4a	N2	M0
	T4b	N0	M0
Stage IIIB	T1	N3b	M0
	T2	N3b	M0
	T3	N3a	M0
	T4a	N3a	M0
	T4b	N1	M0
	T4b	N2	M0
Stage IIIC	T3	N3b	M0
	T4a	N3b	M0
	T4b	N3a	M0
	T4b	N3b	M0
Stage IV	Any T	Any N	M1

Modified from Ref. [99]

[a]If the tumour invades greater or lesser omentum, gastrocolic or gastrohepatic ligaments without breach of peritoneum is classified as T3

[b]Beach of peritoneum corresponds to T4

[c]Intramural extension along alimentary canal into oesophagus or duodenum is not invasion of adjacent organ (ie. Not T4b)

[d]Metastatic carcinoma deposits in subserosal fat with no residual node and no vascular or neural structure are regarded as lymph node deposits

[e]Direct extension into liver, colon, pancreas, diaphragm is classified as T4b, not as M1

[f]Positive peritoneal cytology corresponds to M1

stomach. Mid-portion gastric carcinomas can metastasize into the pancreatic and splenic nodes, whereas lesions of the proximal stomach into mediastinal lymph nodes.

Haematogenous Spread

Even if lymph nodes are not involved, when gastric carcinomas invade the tributaries of the portal venous, spread through the bloodstream occurs and metastases can be commonly seen in liver, followed by the lung, peritoneum, adrenal glands, skin and ovaries.

Sometimes, the distribution of metastases depends on gastric histological type, since gland-forming carcinomas tend to form more likely metastases at liver by haematogenous spread than poorly

cohesive carcinomas, whereas these latter are more likely to give rise to peritoneum and bone metastases than the gland-forming carcinomas [192].

Transperitoneal Spread

Secondary tumour deposits are frequently found in omentum, peritoneum and mesentery. Secondary ovarian deposits are well known as one form of Krukenberg's tumour more frequently associated with diffuse primary signet-ring carcinomas than gland-forming tumours. However, the presence of signet-ring cells within an ovarian mucinous tumour should not automatically exclude the extremely rare primary ovarian tumour.

In the Table 4.3 is reported the updated TNM classification of gastric carcinoma, with clinical and pathological stage groups. Clinical stage groups are different to pathological ones, since they are simplified for nodes, indicating only if they are involved or not, and as a consequence the stage cT4b NX M0, which has poor prognosis, is staged as IV. Furthermore, changes have also been introduced into pTNM groups with respect the previous edition, considering that pT4aN2 and pT4bN0 are now Stage IIIA rather than IIIB.

In addition to clinical and pathological stage, the AJCC also published post preoperative therapy prognostic groups for adenocarcinoma, classified as ypTNM.

Prognosis

Despite the ongoing decrease in morbidity and mortality, gastric cancer continues to be one of the leading types of fatal cancer worldwide. The majority of patients in the West are diagnosed with advanced disease, and only 6–10% of the cases is affected by early-stage cancer [108]. Therefore, in absence of a radical feasible surgery, the prognosis for these patients is poor. The late diagnosis can be due to absence of significant symptoms at an early stage and the lack of validated screening programmes. The expected 5-year survival rate for patients after surgery is approximately 26% in Western countries [193], whereas in Japan, it increases to 50% for T3 tumours and 60–80% for T2 adenocarcinoma [194, 195]. Furthermore, female sex and Japanase ethnicity are positively associated with survival rate as well as higher frequency of EGCs. Accurate staging and surgical expertise have been associated with improved survival in Japan compared to western countries [183, 196, 197]. A relevant feature in resectable cases is represented by complete tumour removal with negative edges [198]. However, despite the resection, it has been observed a local regional recurrence in 40% of the surgical cases and a systematic recurrence in 60% [199–201].

Hereditary Gastric Cancer Syndromes

Familial clustering represents about 10% of gastric cancers and approximately 1–3% arise from inhereted syndromes, which predispose to an incresed risk to develop this pathology [202]. These inherited syndromes include Familial Adenomatous Polyposis (FAP), Lynch syndrome [203–205], Li-Fraumeni syndrome and Peutz-Jeghers syndrome. In FAP patients, the risk to develop gastric cancer is sevenfold higher than the general population [206]. In Lynch syndrome, the high frequency to develop gastric carcinoma in earlier age than sporadic neoplasm is related to germline mutations of hMLH1 and hMSH2, genes of DNA mismatch repair (MMR) [203, 204, 207], as well as of TP53 in Li-Fraumeni syndrome [208]. Even though gastrointestinal cancer represents less than 10% of malignancies associated with syndrome, 50% of the cases are gastric cancer. Recently, it has also been reported that frameshift mutations in the STK11 gene in Peutz-Jeghers patients are responsible to the development of aggressive gastric cancers [209]. Moreover, a novel germline mutation of the LKB1 gene has been reported in a patient with sporadic Peutz-Jeghers syndrome with early onset of gastric cancer [210].

Criteria for Familial Gastric Cancer

Familial gastric cancers can be divided on the basis of knowledge of histopathology of the tumours: in absence of hystopathological characterization of carcinomas of individuals with familial aggregation, the carcinomas are simply referred to as familial gastric cancer (FGC), whereas when the histopathology of one or more

neoplasms of individuals of familial aggregation are available, gastric cancers can be distinguished in hereditary diffuse gastric cancer (HDGC), familial diffuse gastric cancer (FDGC) and familial intestinal gastric cancer (FIGC).

Hereditary Diffuse Gastric Cancer (HDGC)

With this definition is indicated an autosomal-dominant syndrome associated with the development of signet-ring cell (diffuse) gastric cancer and lobular breast cancer. Germline mutations in the E-cadherin gene CDH1 are the genetic basis of the HDGC as discovered by Guilford in 1998 [211].

The International Gastric Cancer Linkage Consortium (IGCLC) defined families with HDGC syndrome on the basis of the meeting with one of the following clinical criteria:

1. Two or more documented cases of diffuse gastric cancer in first or second-degree relatives with at least one being diagnosed before the age of 50 years.
2. Three or more cases of documented diffuse gastric cancer in first- or second-degree relatives, independent of age of diagnosis [212].

Women of these mentioned families have an elevated risk of lobular breast cancer [213–217].

In 2010, the IGCLC criteria for genetic testing have been updated as reported in Table 4.4.

An alternative genetically-based nomenclature has been proposed, that restrains the term "HDGC" only to the families with germline mutations in the CDH1 gene [211, 218].

Table 4.4 Selected criteria for the genetic screening of suspected HDGC families

2 or more cases of diffuse gastric cancer in first or second-degree relatives, of which at least one diagnosed under the 50 years age
3 or more cases of confirmed diffuse type of gastric carcinoma in first- or second-degree relatives, independent of age of diagnosis
1 case of diffuse gastric cancer occurring before 40 years age, without a family history
Personal or family history of diffuse gastric carcinoma and lobular breast cancer, of which one diagnosed under the age of 50 years

In the clinically defined HDGC, mutations in the CDH1 gene have been reported in 30–40% of cases [216, 219, 220], most of which are truncating mutations and, in a minor extent, missense mutations [221, 222]. In absence of point mutations, large germline deletions have been described in 6.5% of HDGC families [220]. Furthermore, germline mutations can occur in the whole gene lenght of CDH1 and no hot spots have been identified.

A second CDH1 hit is required to initiate diffuse gastric carcinoma in mutant carriers. Most frequently, this occurs via promoter hypermethylation (epigenetic modification), and less frequently via CDH1 mutations and loss of heterozygosity (LOH) [223–225].

Because 60–70% of patients with HDGC are negative for CDH1 germline mutations, the attention is focused on the search for additional genes involved in HDGC.

Molecular Aspects of Gastric Carcinoma

The genomic changes involved in the multistep process of gastric carcinogenesis are the result of genetic and epigenetic abnormalities including (1) genomic instability through two distinct pathways: microsatellite instability (MSI) and chromosomal instability; (2) epigenetic alterations and (3) silencing of tumour-suppressor genes and activation of oncogenes.

Microsatellite Instability (MSI)

MSI is caused by defects in the DNA mismatch repair (MMR), a system able to recognize and correct nucleotide mismatches occurring during DNA replication. The MMR machinery consists of the MLH1, PMS2, MLH2, and MLH6 proteins.

Gastric cancers with MSI often show epigenetic silencing of the mutL homolog 1 (MLH1) gene. As member of MMR, when expressed, MLH1 protein plays an essential role in DNA mismatch repair and is responsible for fixing errors that occur during DNA replication. Furthermore, MSI-positive gastric cancers are

often associated with activation of the epidermal growth factor receptor (EGFR) and PI3K pathways [226].

MSI has been detected in early stages of carcinogenesis including chronic gastritis, IM, dysplasia, and adenoma and in 15–49% of sporadic gastric cancers [227]. In gastric carcinomas, MSI is observed in 5–10% in diffuse type and in 15–40% of intestinal type.

Chromosomal Instability

About 80% of sporadic adenomas show chromosomal instability, which results in altered DNA copy numbers (aneuploidy) and various changes in chromosome regions, such as translocation, amplification, deletion or the loss of heterozygosity (LOH) [80]. Contrary to MSI, the mechanism underlying chromosomal instability is not well known. Aneuploidy results from alterations in mitotic segregation and centrosomal abnormalities [228]. Mechanisms and genes involved in aneuploidy have been reviewed by Aguilera and Gomez-Gonzalez [229].

Tumours characterized by chromosomal instability are frequently associated with activation of the RTK/RAS pathway and EGFR, HER2, HER3, JAK2, FGFR2, MET, PIK3CA and KRAS/NRAS amplifications [230].

Epigenetic Alterations

Several studies have proved that epigenetic alterations affected the cancer-related genes (i.e. APC, KRAS, TP53, hMLH1, CDKN2A/p16) even more commonly than genetic mutations [231–233]. Hypermethylation associated with gene silencing occurs at specific sites of the promoter sequences, defined as CpG islands [234]. The simultaneous hypermethylation of CpG island of multiple genes is referred to as CpG island methylator phenotype (CIMP). An increasing frequency of promoter methylation involving multiple genes has been shown to occur in the progression from chronic gastritis to carcinoma [235]. Tumours with multiple concurrently hypermethylated *loci* are described as high-CIMP. High-CIMP is frequently found in MSI-positive gastric cancers, and is associated with hypermethylation of MMR genes (hMLH1), as previously reported [232]. The CIMP phenotype is thought to be an early event in gastric cancer. Its presence in adjacent normal tissue may be associated with *H. pylori* infection, which points to the possible mechanism of its contribution to gastric carcinogenesis [232].

Moreover, recent studies revealed that CIMP is more prevalent in the diffuse as opposed to the intestinal type of gastric cancer [236, 237].

Tumour-Suppressor Genes

Several tumour-suppressor genes have been reported in the development of gastric cancers, such as CDH1 [238–242] and RB1 [243] in diffuse-type carcinomas and APC [244–247] and DCC [248, 249] in intestinal-type carcinomas. In this regard, somatic mutations in the APC gene are present in 6% of IM and in 20–40% of gastric adenomas and therefore are also considered as an early event in gastric carcinogenesis [3, 227].

Other genes such as PTEN and TP53 are deregulated in both types of gastric carcinoma, even though alteration of TP53 has been seen to be more common in intestinal-type carcinoma [246, 250–253]. As it has been reported for APC gene, alterations in TP53 were found in at least 30% of regions of IM, and in 33–58% of gastric dysplasia and adenomas, indicating that mutations in TP53 is an early event in gastric carcinogenesis [227].

Oncogenes

In intestinal type of gastric cancer, some oncogenes such as HER2 [254–256] and KRAS [257–260] are preferentially altered. Amplification and/or overexpression of HER2, a member of the human tyrosine kinase receptor family, is detected in 7–34% of gastric adenocarcinomas [39]. The overexpression of HER2 seems to occur in early event of gastric carcinogenesis, since its expression rises significantly from low-grade dysplasia to high-grade dysplasia to adenocarcinoma [261]. The increasing interest for HER2 expression in gastric carcinoma by immunohistochemical (IHC) and *in situ* hybridization assays is due to the positive responses of these neoplasms to treatment with Trastuzumab, a humanized monoclonal antibody targeting the HER2 receptor, as

demonstrated by ToGA trial [262]. In this regard, in contrast to HER2 expression in breast cancer, HER2 immunohistochemical expression in gastric cancer is more heterogeneous and U shaped or lateral staining is more frequent in gastric cancer rather than a complete staining [263, 264].

Since the correlation between HER2 amplification and protein overexpression in gastric cancer is less stringent than in breast carcinoma [263], the European Medicines Agency (EMA) recommends to evaluate HER2 as first by immunohistochemistry, followed by fluorescence in situ hybridization (FISH) in IHC2-positive cases [265].

KRAS mutations have been detected in more than 50% of intestinal carcinoma, but not in diffuse carcinoma. Activating KRAS mutations result in RAS proteins that are constitutively active, leading to stimulation of downstream signalling pathway independent of EGFR signalling. EGFR is another member of the human tyrosine kinase receptor family that has been shown to be overexpressed by immunohistochemistry in 27% of gastric cancers, whereas gene amplification by FISH was evident in less than 3% of more than 500 cases of GC tissue analysed [266].

Differently to intestinal type, other oncogenes are preferentially altered in diffuse gastric carcinoma, among which BCL2 [267, 268] and FGFR2 [269, 270]. Oncogenes including CTNNB1 (encoding β-catenin) [271], MET [272] and MYC [273, 274] are deregulated both in intestinal and diffuse gastric cancers. Additionally, genes involved in the cell cycle regulation such as CDKN1B [275–277] and cyclin E [278], have been also reported to be altered in gastric cancer.

Conclusion

About 90–95% of gastric cancers (GC) are adenocarcinomas. These cancers develop within the cells of the mucosa, the inner most lining of the stomach. Other gastric cancer histotypes are lymphoma, gastrointestinal stromal tumours (GISTs), carcinoid tumours and other rare tumours.

The accurate and precise classification of gastric epithelial tumours is the prerequisite to better understanding the biology of this tumoural entity that deserves increasing attention due to early diagnosis reliability and more efficacious multimodal therapeutic approaches.

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5 Diagnostic, Prognostic, Predictive and Therapeutic Tissue Biomarkers in Gastric Cancer

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Introduction

Gastric cancer is one of the most common lethal cancers worldwide. Every year worldwide, there are 723,000 cancer-related deaths caused by gastric cancer according to the World Health Organization (WHO). It is the third leading cause of death by cancer and the fifth most common cancer in the world [1].

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_5

The incidences and mortality rates are higher in men than in women.

Stomach cancer is common in different parts of the world, including Europe, the United States, Korea, China and Japan [2].

There are different factors that increase the risk of gastric cancer. They can be divided into genetics factors, such as mutations of the BRCA1 and BRCA2 genes, and non-genetic factors, such as age, sex, family history, smoking, alcohol consumption, obesity, physical inactivity, stress and infections. A relevant role has been recognized for *Helicobacter pylori* infection in the onset of gastric cancer [3, 4].

Despite progress in the diagnosis and treatment of advanced gastric cancer, the prognosis remains poor, mainly due to difficulty in diagnosing the disease in its early stages. Patients diagnosed with advanced GC have a dismal prognosis with a high mortality rate. Therefore, early detection of cancer can reduce the probability of disease progression, advanced cancer and death and increase the chances of treatment success.

In this chapter, the classification of gastric cancer into various histological subtypes and their biological characteristics, disease predictions by immunohistochemistry and in situ hybridization and gastric cancer assessments with standard and new tissue biomarkers are discussed.

Gastric Carcinoma

Precursor Lesions

Gastric Intestinal Metaplasia (GIM)

Gastric intestinal metaplasia (GIM) (Fig. 5.1) is an intermediate precancerous gastric lesion in the gastric cancer cascade of chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and adenocarcinoma. Although the risk of gastric cancer is increased in patients with intestinal metaplasia, the absolute risk is low. GIM can be divided into two subtypes: the complete type (type I) that morphologically resembles the normal small intestinal mucosa with absorbing cells, Paneth cells and goblet cells; the incomplete sulphomucin-negative type (type II), consisting of goblet cells scattered among gastric foveolar and neck cells; and the incomplete sulphomucin-positive type (type III), showing goblet cells scattered among sulphomucin-producing columnar cells. Another classification has been proposed by Tatematsu et al., in which IM can be divided into gastric-and-intestinal mixed (GI) and solely intestinal (I) types [5], expressing different tissue markers, so that IM might also be evidenced by the progressive intestinalization of stem cells from the GI-type to I-type. In this context, precocious identification of the early stages of GIM may be achieved with LI cadherin, (liver-intestine (LI) cadherin or CDH17, see below) which has higher sensibility and specificity than villin. However, specific subsets of patients with intestinal metaplasia might have a greater risk of progression. Hence, it is necessary to identify new biomarkers to better identify high-risk subgroups and to determine the optimal interval for surveillance in patients at increased risk for gastric cancer.

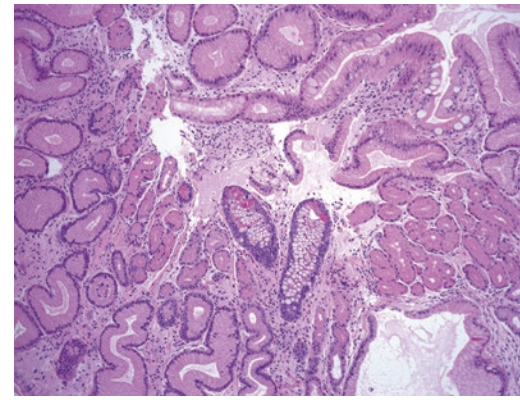

Fig. 5.1 Gastric intestinal metaplasia (GIM). Original Magnification 100×

Gastric Epithelial Dysplasia (GED)

Gastric epithelial dysplasia (GED) refers to neoplastic noninvasive proliferation widely accepted as a precursor to gastric adenocarcinoma. The frequency of GED increases with age, especially in men in their fifth decade of life or above. This tendency may be related to atrophic changes, especially intestinal metaplasia of the gastric

mucosa, among the elderly. The prevalence of GED shows considerable geographic differences, and it seems to be associated with the regional prevalence of *Helicobacter pylori* infection [6].

Based on its morphological characteristics, GED is divided into three subtypes: low-grade, high-grade and indefinite dysplasia. It seems that 15% of low-grade dysplasia can evolve to carcinoma, while high-grade dysplasia has an 85% chance of progression (Fig. 5.2).

Gastric Adenocarcinoma (GA)

Gastric adenocarcinoma (GAC) is the most common histological type (~95%) of all malignancies originating in the stomach. It is a heterogeneous disease with different histological characteristics (phenotypes) and genotypes.

According to the most recent WHO classification (Bosman et al., 2010), GAC can be divided into five main types: papillary adenocarcinoma, tubular adenocarcinoma, mucinous adenocarcinoma, poorly cohesive carcinoma and mixed adenocarcinoma [7]. In addition, the Lauren classification divides GC into four histological types: intestinal gastric, diffuse gastric cancer, mixed and indeterminate types with distinct clinicopathological features. Intestinal gastric cancer is more associated with environmental factors such as infection of *H. pylori*, a high-salt diet, smoking and obesity [8], while diffuse gastric cancer comprises non-cohesive cells and is more commonly observed in younger patients, with an apparent hereditary feature. It has been reported that approximately 10% of gastric cancer cases show familial clustering [9]. The diffuse type of GA usually develops de novo and is usually not associated with *H. pylori*.

Rare hereditary forms of GAC are associated with germline mutations in various genes, such as CDH1, which encodes the tumour suppressor and cell adhesion protein cadherin 1 (also known as E-cadherin). Additionally, impaired function in mismatch repair genes (such as MLH1) or in CTNNA1 (which encodes catenin α1, a cell adhesion protein) or inactivating mutations in BRCA genes (which encode DNA damage repair proteins) can increase the risk. Furthermore, infection with *Helicobacter pylori* and Epstein-Barr virus (EBV), as well as exposure to other carcinogens, is known to contribute to the development of GAC [10] (Figs. 5.3 and 5.4).

Tissue Biomarkers of Gastric Cancer

The search for various antigens is critical in the diagnosis, prognosis and prediction of cancer. Different subtypes of gastric carcinoma show different antigenic patterns. Recently, several mole-

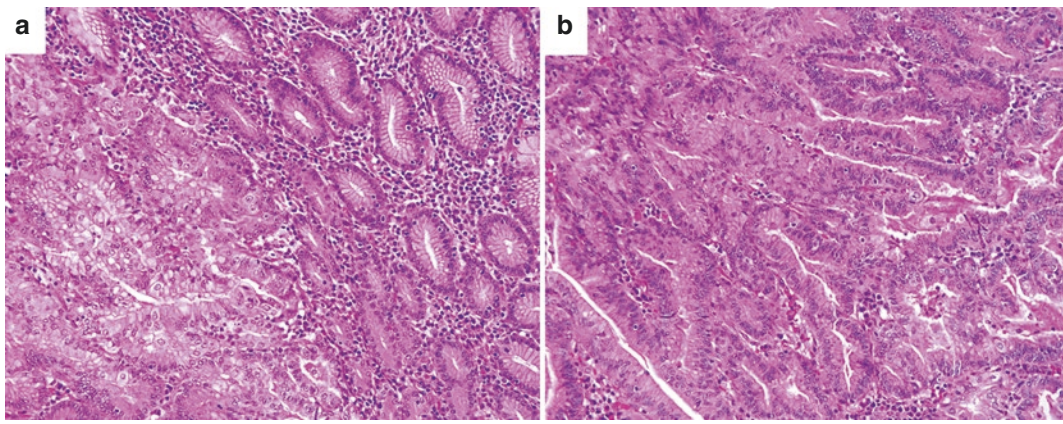

Fig. 5.2 Gastric epithelial dysplasia (GED): (**a**) Transition from normal tissue (right) to high-grade glandular dysplasia (left). Original magnification 200×; (**b**) High-grade dysplasia. Original magnification 200×

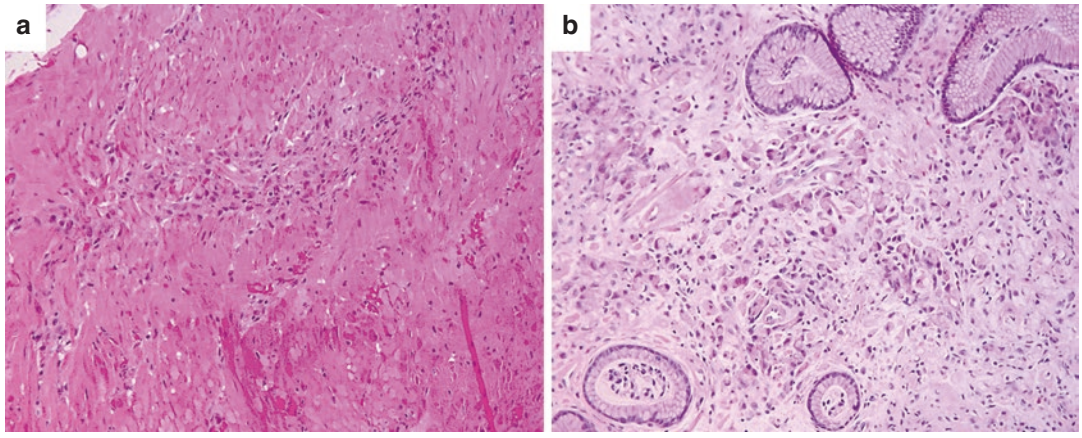

Fig. 5.3 Gastric adenocarcinoma (GA): (**a**) Diffuse-type gastric adenocarcinoma with infiltration of the muscular wall, original magnification 200×; and (**b**) neoplastic infiltration of lamina propria, original magnification 200×

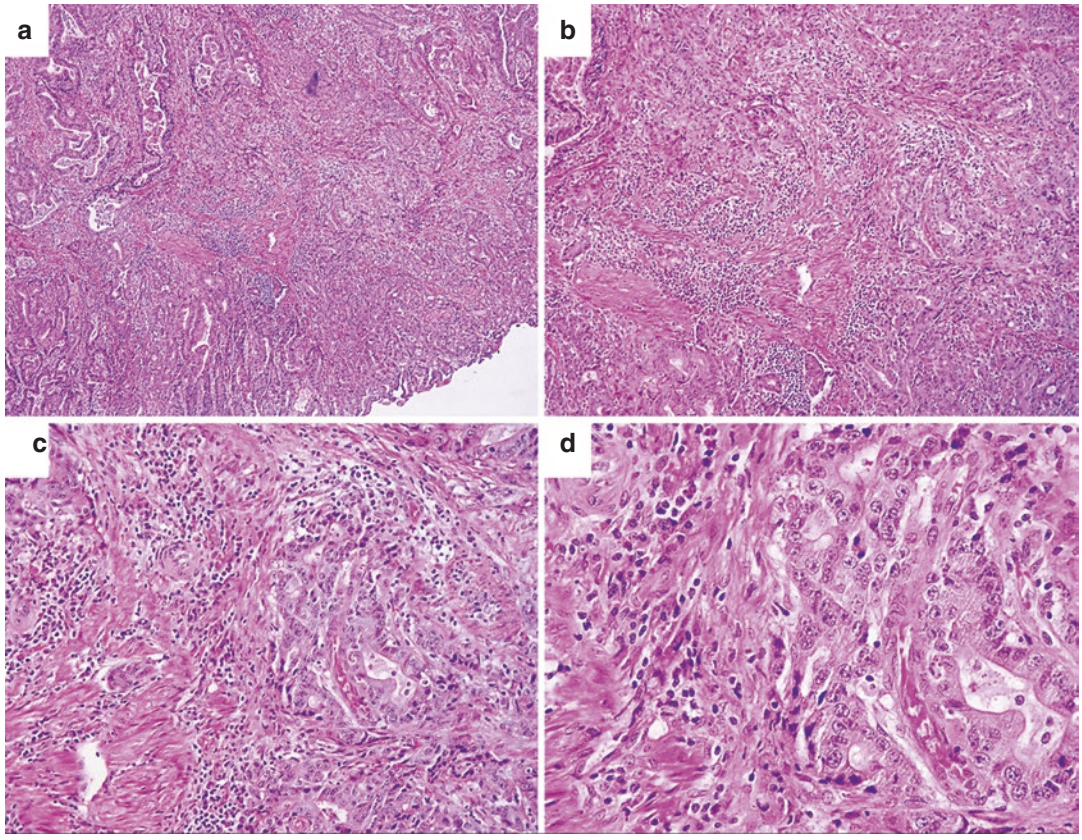

Fig. 5.4 Tubulo-papillary adenocarcinoma of the stomach at different original magnifications. (**a**) 50×; (**b**) 100×, (**c**) 200×; (**d**) 400×

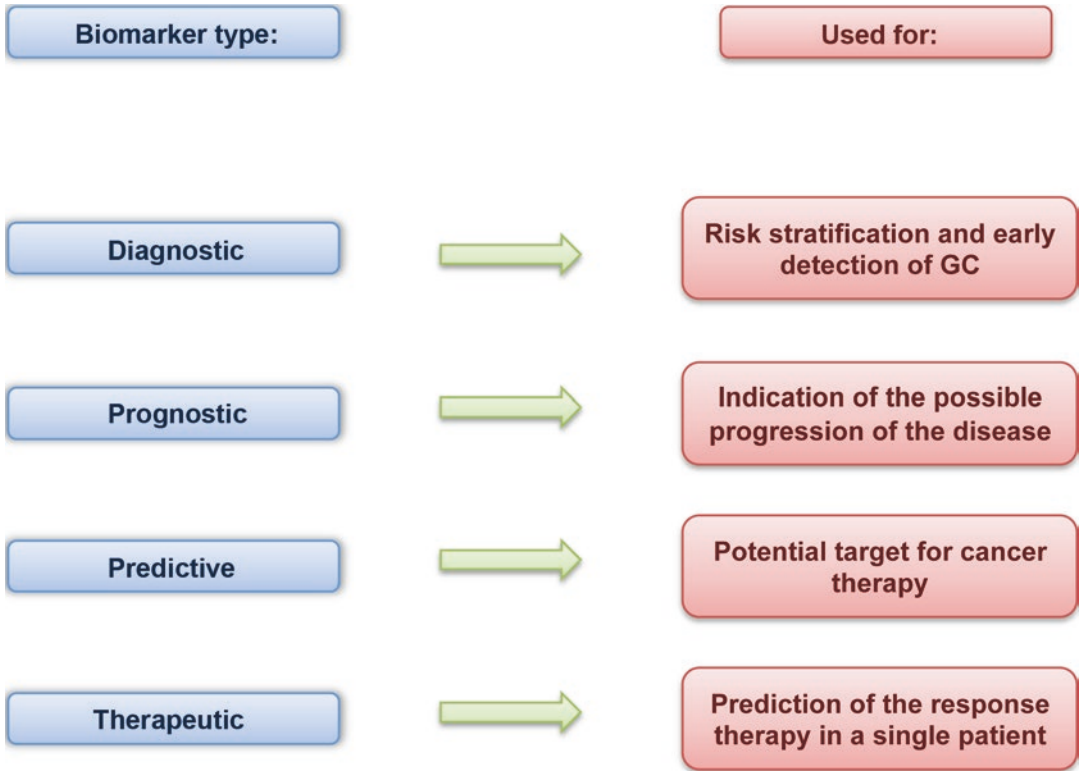

Fig. 5.5 Biomarker types and their utilization in clinical practice

cules have been proposed as novel biomarkers. They can be classified into four types: diagnostic, prognostic, predictive and therapeutic (Fig. 5.5). Notably, some markers might have an inclusive theranostic multirole in gastric cancer.

Diagnostic Tissue Biomarkers

Cytokeratins

Cytokeratins (CKs) are keratin proteins found in the intracytoplasmic cytoskeleton of epithelial tissue. They are important components of intermediate filaments, and they are involved in fixation of the nucleus and maintenance of cell morphology. There are at least 20 known cytokeratins, and they can be expressed differentially in healthy and neoplastic tissues. Thus, they are useful diagnostic tools.

Gastric adenocarcinomas stain with low- and high-molecular-weight cytokeratins (L-HMWCKs).

CK7 is expressed in approximately 80% of gastric adenocarcinomas, and it is observed in various ductal epithelial cell carcinomas, arising in the pancreatobiliary tract and renal collecting ducts. Otherwise, CK20 expression is reported in approximately 40% of gastric adenocarcinomas in a patchy or diffuse distribution. CK20 is specific to certain types of cancer and is typically used in combination with CK7 to distinguish different types of tumours (Fig. 5.6). However, the CK7/CK20 coordinate staining pattern has been considered of little utility for the differential diagnosis of primary gastric adenocarcinoma versus

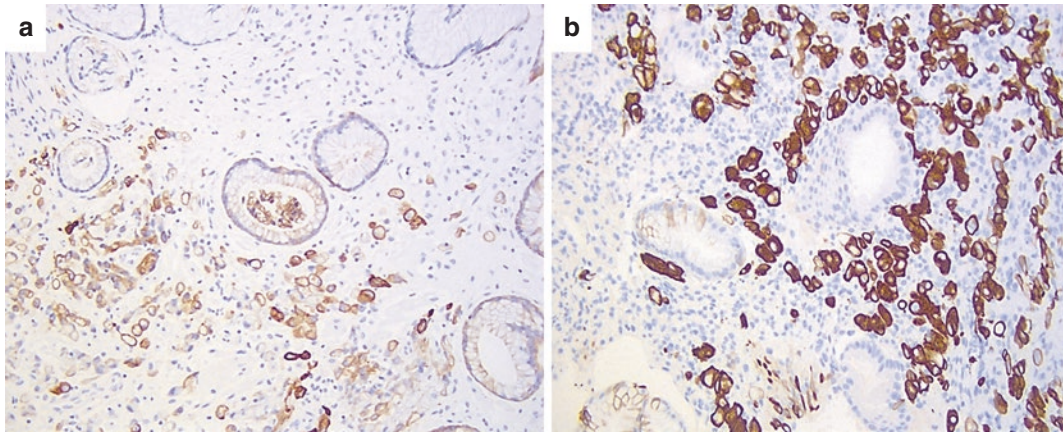

Fig. 5.6 (**a**) Immunohistochemical expression of CK7 in tubulo-papillary adenocarcinoma, original magnification 200×; (**b**) immunohistochemical expression of CK20 in tubulo-papillary adenocarcinoma, original magnification 200×

Fig. 5.7 Coordinate immunostaining pattern in gastric adenocarcinoma. Its use is not useful in the differential diagnosis with other adenocarcinomas

metastatic adenocarcinomas to the stomach (Fig. 5.7).

Other cytokeratins, strongly expressed in gastric adenocarcinomas, are CK18 and CK19. There is evidence that CK18 is involved in the invasion, growth and metastasis of tumours. Therefore, it can be used as a biomarker of gastric carcinoma aggressiveness [11]. Moreover, CK8 and cytokeratin Cam.5.2 are strongly expressed in gastric adenocarcinoma, whereas CK17 expression is constantly negative [12].

CDX2

CDX2 is a homeobox gene that encodes an intestine-specific transcription factor, and it is expressed in the nuclei of epithelial cells throughout the GI tract. It is considered a tumour suppressor gene in different tumour types, including gastric adenocarcinoma, where it is variably

expressed (positivity ranges from 20% to 90%), and its expression is heterogeneous in diffuse-type cancers compared with strong and diffuse staining in tubular adenocarcinomas.

CK7 and the CK20 or CDX2 markers constitute an important panel for the diagnosis of GI tumours versus tumours of unknown origin. However, in some cases, the patterns of expression may vary, and the presence or absence of these markers may not lead to a definitive diagnosis [13] (Fig. 5.8).

Mucin Core Polypeptides (MUC)

Mucin core polypeptides (MUC) are high-molecular-weight glycoproteins expressed throughout the gastrointestinal tract, with a key role in mucosal protection and function. They are responsible for the mucus gel layer, which covers the mucosa. Immunohistochemistry specific to various mucins (MUC1, MUC2 MUC5AC and MUC6) has been used to evaluate the mucin phenotypes of gastric cancer. MUC1 is normally expressed by enterocytes and intestinal goblet cells, and it is present in rare cases; MUC2 is normally secreted by intestinal goblet cells, and it is expressed in approximately 50% of cases; MUC5AC is expressed by gastric foveolar mucus cells and neoplastic goblet cells, but it is positive in 38–70% of cases; MUC6 is secreted by gastric antral and fundic

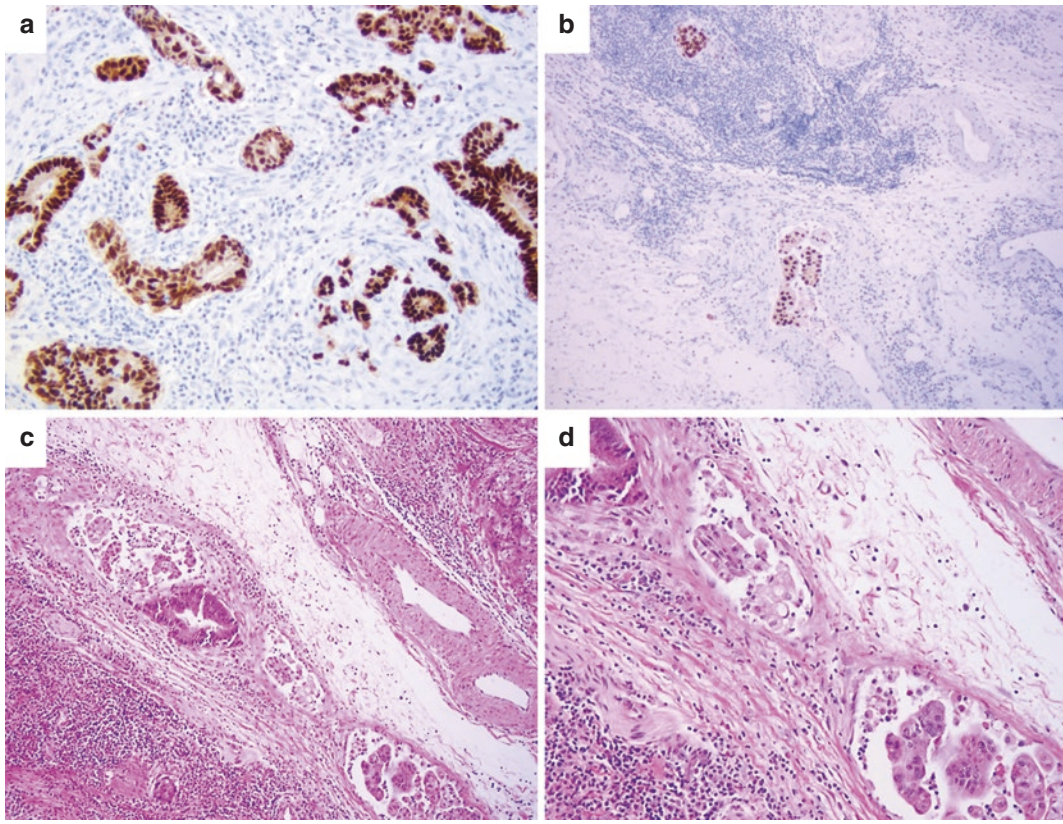

Fig. 5.8 (**a**) Immunohistochemical expression of CDX2 in a gastric tubulo-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain); (**b**) immunohistochemical expression of CDX2 with vascular invasion in a gastric tubulo-papillary adenocarcinoma, original magnification 100× (haematoxylin counterstain); (**c**) vascular invasion in a gastric tubulo-papillary adenocarcinoma (H&E), original magnification 100×; (**d**) same case at higher magnification (H&E), 200×

gland cells, and it is positive in 30–40% of cases [14] (Fig. 5.9).

β-catenin

β-catenin is an 88-kD member of the catenin family of proteins, which are important constituents of the cytoskeleton. It regulates gene expression and is an important component of the Wnt signalling cascade. Aberrant activation of the Wnt/β-catenin signalling pathway is involved in the development and progression of gastric cancer. The accumulation of β-catenin in the cytoplasm causes abnormal translocation to the nucleus and defects in gene expression. Thus, it seems that it is involved in tumour initiation, tumour growth, metastasis and resistance to therapy [15] (Fig. 5.10).

Chromogranin A and Synaptophysin

Chromogranin A and synaptophysin are two neuroendocrine markers. Chromogranin A is a member of the granin family of neuroendocrine secretory proteins. It is widely distributed in the secretory granules of most polypeptide-producing endocrine tissues and is considered very useful as a diagnostic aid for neuroendocrine normal and tumour cells. It is the most valuable marker of neuroendocrine tumours and is highly specific but less sensitive than synaptophysin. Synaptophysin is an integral membrane glycoprotein that was originally isolated from bovine neuronal presynaptic vesicles and is considered a significant neuroendocrine marker. Both chromogranin and synaptophysin play roles in characterizing some subsets of common gastric adenocarcinoma with expression of neuroendocrine markers (Fig. 5.11) [16].

Cyclooxygenase 2 (COX2)

Cyclooxygenase 2 (COX2) is an enzyme encoded by the PTGS2 gene; in humans, only one isoform is present. It is involved in the conversion of arachidonic acid to prostaglandin H2, an important precursor of prostacyclin. The expression of COX-2 in gastric cancer is upregulated, and its molecular mechanisms have been investigated. COX-2 likely plays a role in the promotion of proliferation in GC cells while inhibiting apoptosis, assisting angiogenesis and lymphatic metastasis and participating in cancer invasion and immunosuppression. Different studies have suggested that COX-2 overexpression is not related to the clinicopathological characteristics of gastric cancer patients but is related to tumour node metastasis clinical stage, depth of invasion and metastasis [17, 18]. Furthermore, it seems that COX-2 protein expression is associated with the intestinal histological subtype, tumour size, proximal location, advanced clinical stage and lymph node involvement. Thus, it seems likely that COX-2 plays a role in early gastric carcinogenesis [19, 20]. *Helicobacter pylori* infection, tumour sup-

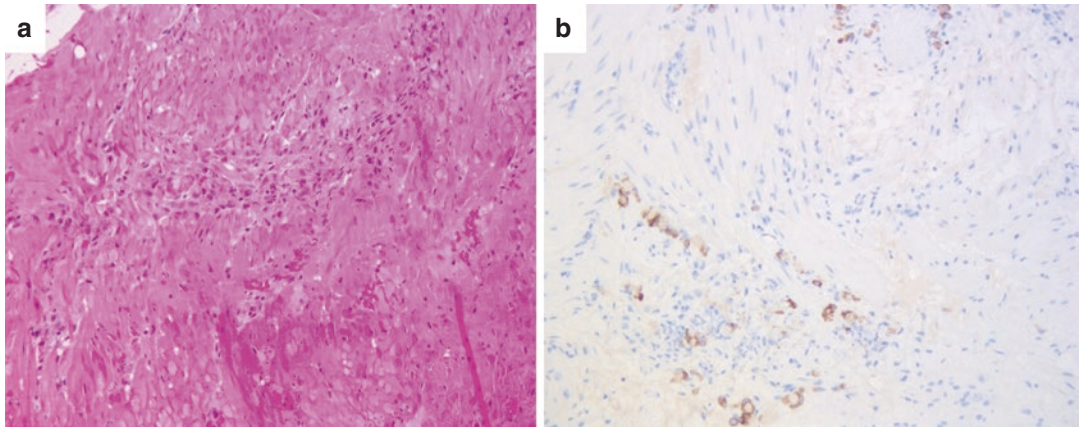

Fig. 5.9 (**a**) Diffuse-type adenocarcinoma (H&E), expressing; (**b**) MUC1, original magnification 200× (haematoxylin counterstain)

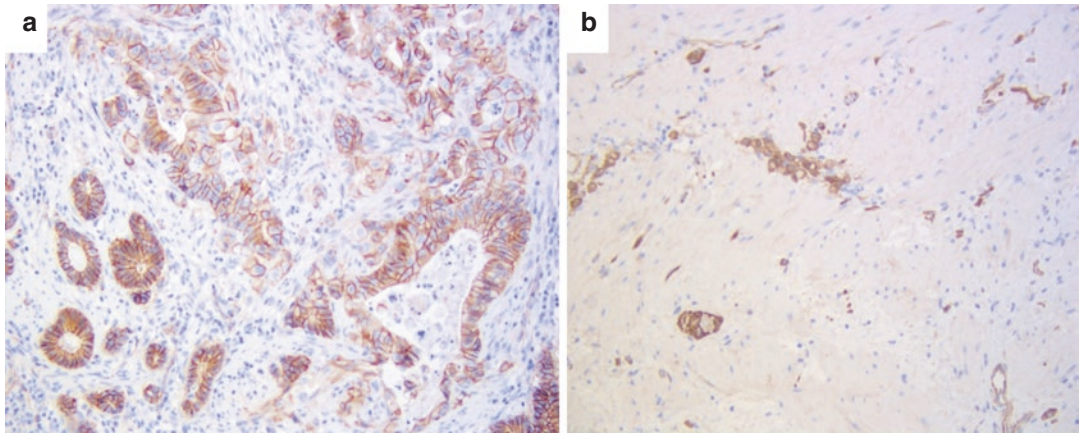

Fig. 5.10 (**a**) Immunohistochemical expression of β-catenin in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain); (**b**) immunohistochemical expression of β-catenin in diffuse-type gastric adenocarcinoma, original magnification 200× (haematoxylin counterstain)

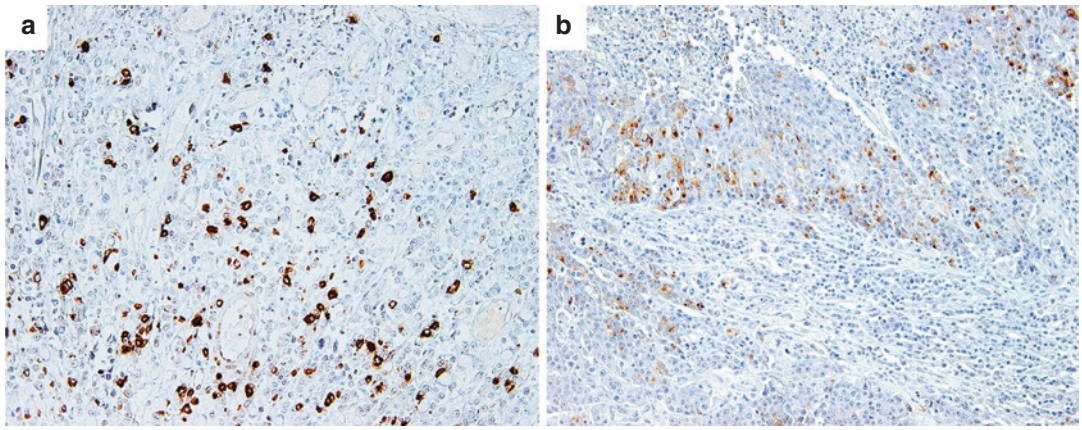

Fig. 5.11 (**a**) Immunohistochemical expression of chromogranin A in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain). (**b**) Immunohistochemical expression of synaptophysin in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain)

pressor gene mutation and activation of nuclear factor-kappa B may be responsible for the elevated expression of COX-2 in gastric cancer [21].

Carcinoembryonic Antigen (CEA)

In adults, CEA is expressed only in cancer cells, primarily adenocarcinomas, and may be used for diagnostic purposes. It is a set of highly related glycoproteins that are involved in cell adhesion. CEA is usually produced in the gastrointestinal tissue during the development of the foetus and terminates before birth. In gastric cancer, CEA has been used to distinguish between this and other similar types of cancers. Because of the possibility of cross-reactivity, false-positive results are observed, and this assay is typically used in combination with other analyses.

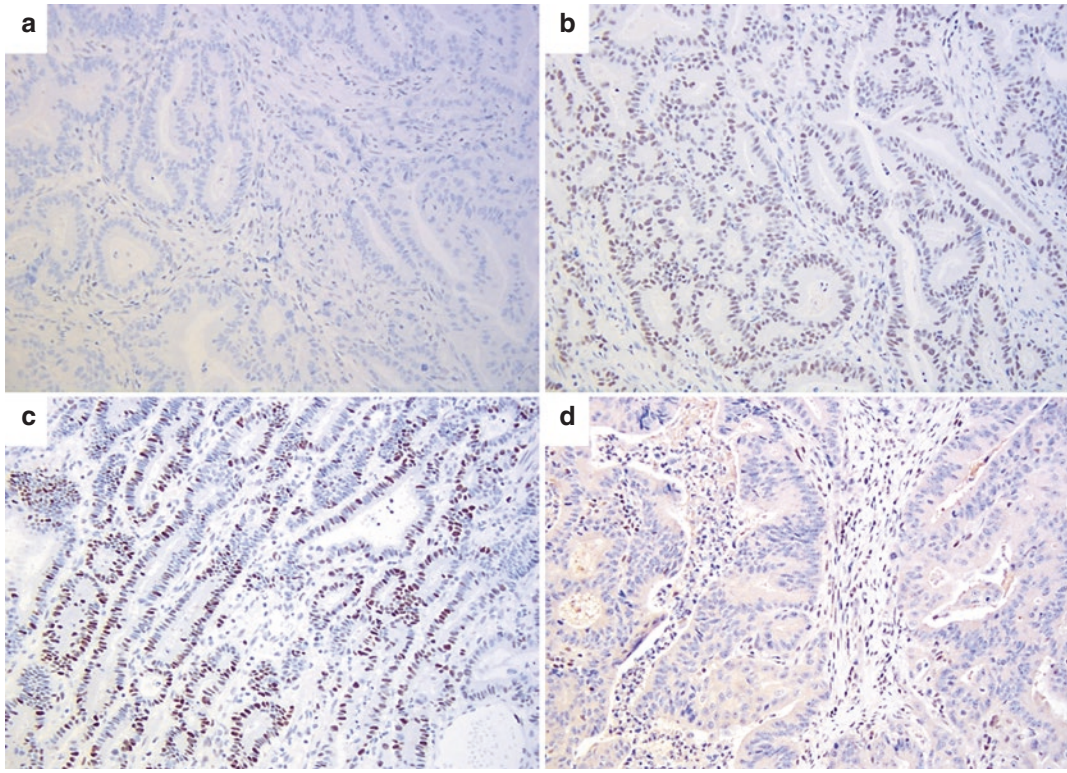

Fig. 5.12 (**a**) Immunohistochemical expression of MLH1 in a gastric tubular-papillary adenocarcinoma, negative case original magnification 200× (haematoxylin counterstain); (**b**) immunohistochemical expression of MSH2 in gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain); (**c**) immunohistochemical expression of MSH6 in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain); (**d**) immunohistochemical expression of PMS2 in a gastric tubular-papillary adenocarcinoma, negative case, original magnification 200× (haematoxylin counterstain)

DNA Mismatch Repair Proteins MLH1, MSH2, MSH6 and PMS2

DNA mismatch repair (MMR) genes encode proteins that detect and repair DNA mismatches that can occur during cell replication. Mutations in any of the MMR genes MSH2, MLH1, MSH6 or PMS2 increase the risk of different types of gastric cancer. These proteins function as heterodimers; MLH1 associates with PMS2, and MSH2 associates with MSH6 [22] (Fig. 5.12).

Prognostic Tissue Biomarkers

P53 Protein

P53 is a nuclear protein that functions as a transcriptional factor whose duty is to maintain genomic stability. When DNA damage occurs, p53 binds to the DNA and activates the transcription of genes responsible for stopping the cellular cycle and causing apoptosis of the cell. p53 is encoded by the gene TP53 located on chromosome 17p13.1. It is a tumour suppressor gene that is inactivated in the development of many malignancies, including gastric cancer. The expression rate of p53 detected by immunohistochemistry is reported to be 13–54% in gastric cancer [23].

Mutations in TP53 lead to nuclear staining due to the accumulation of mutant p53, which is resistant to degradation. A cell without the mutation does not show immunohistochemical staining of p53 because there is no such accumulation in the cell [24].

The prognostic role of p53 expression in gastric cancer has been searched in many studies, but it is controversial. In some studies, it has been

suggested that patients without p53 expression have a longer survival, and p53 is a bad prognostic factor [25, 26].

Other studies report a correlation between p53 overexpression and the size of the gastric tumour [27].

The association between p53 overexpression with lymph nodes, metastasis and shorter survival remains controversial because it has been reported in some studies but not in others. Therefore, to date, p53's role as a prognostic marker needs to be confirmed [28] (Fig. 5.13).

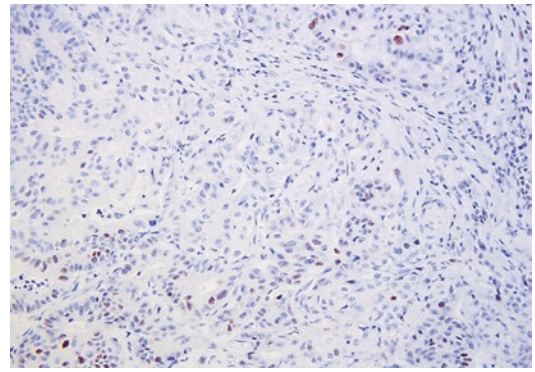

Fig. 5.13 Immunohistochemical expression of P53 in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain)

Carcinoembryonic Antigen (CEA)

In most cases, the CEA level is low in the blood of healthy individuals. Serum and tissue CEA is a representative tumour marker that has been known to be elevated in almost all solid tumours, mostly colorectal cancers, but also in gastric cancers and other neoplastic tissues. Therefore, it can be considered a negative prognostic factor [29].

Many studies have shown that increased preoperative serum CEA levels are associated with an increased risk of recurrence and a poor prognosis, and the prognostic effect of the serum CEA level is independent of the tumour-node-metastasis stage; thus, it can be considered a prognostic marker of gastric cancer. Otherwise, the expression of CEA in tissues is unclear and still under study. It is likely related to the serum expression of CEA, but is not correlated with the size or location of the primary tumour [30] (Fig. 5.14).

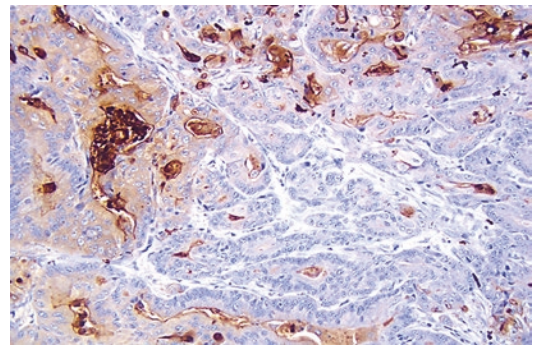

Fig. 5.14 Immunohistochemical expression of CEA in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain)

E-Cadherin

E-cadherin (epithelial-cadherin) is a transmembrane glycoprotein encoded by the CDH1 gene located on chromosome 16 (q22.1). It plays a crucial role in calcium-mediated adhesion, the differentiation of epithelial gastric cells and the prevention of neoplastic transformation. CHD1 has been reported to be one of the most important suppressor genes in gastric cancer, and its inactivation leads to an increase in the proliferation, invasion and metastasis of tumour cells. Different mechanisms can lead to the downregulation of E-cadherin, including mutations in the CDH1 gene, loss of heterozygosis (LOH), silencing by a suppressor that binds the gene promoter or hypermethylation and microRNAs that control E-cadherin expression. Furthermore, germline mutations in the CDH1 gene can cause hereditary diffuse gastric cancer (HDGC) [31].

It seems that different alterations lead to various clinical manifestations and histotypes, and there is an association between abnormal protein expression, tumour grade and metastases to regional lymph nodes. Therefore, E-cadherin can be considered a prognostic biomarker, associated with a worse prognosis and lower survival rate.

It can also be considered a predictive biomarker of the sensitivity to a specific therapy. In particular, its impairment reduces the response to

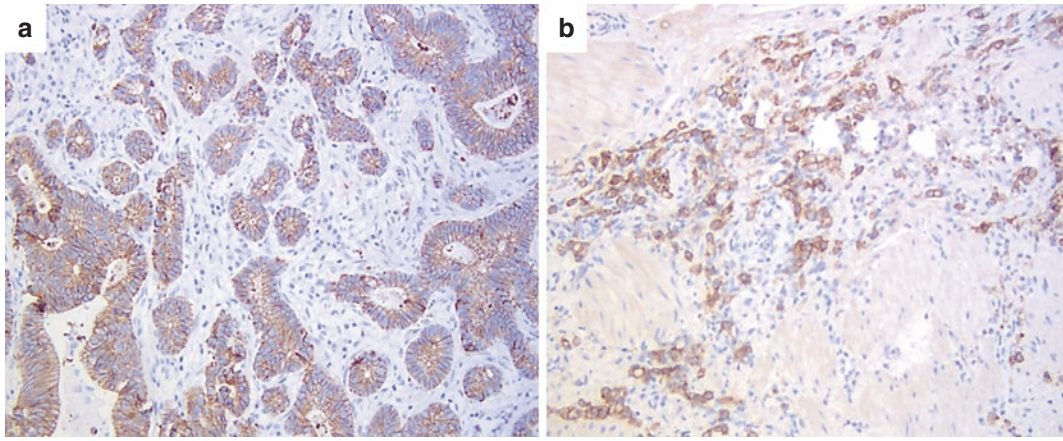

Fig. 5.15 (**a**) Immunohistochemical expression of E-cadherin in a gastric of tubular-papillary adenocarcinoma original magnification 200× (haematoxylin counterstain). (**b**) Immunohistochemical expression of E-cadherin in a gastric diffuse-type adenocarcinoma original magnification 200× (haematoxylin counterstain)

both conventional and targeted therapies. Therefore, it could be important to identify CDH1 mutations at the moment of the diagnosis to predict whether that cancer is going to be responsive to therapy so it could help in choosing a more suitable therapy for a specific patient [32] (Fig. 5.15).

EGFR

EGFR belongs to the family of tyrosine kinase receptors. EGFR overexpression, which is observed in 27–44% of gastric cancer patients, has been generally reported to be a poor prognostic factor. It is probably related to a cancer histology of slight differentiation, low survival and high stage, but there are still many doubts about this correlation [33, 34]. EGFR seems to have no predictive value. Indeed, the use of anti-EGFR (cetuximab and panitumumab) associated with chemotherapy does not show any improvement in clinical outcome.

FGFR

The FGFR/FGF cascade is a complex intracellular pathway that controls cellular proliferation and tumour growth, angiogenesis and dissemination. Four members of the FGFR family, FGFR1, FGFR2, FGFR3 and FGFR4, have been identified. These receptors are different depending on their ligand, binding affinity and tissue distribution [35].

Genetic modification or overexpression of FGFRs has been associated with the initiation and progression of several tumour types due to gene amplification, translocation and mutations, leading to enhanced kinase activity. *FGFR* gene abnormalities have been reported in various cancers, including stomach cancer. In particular, in gastric cancer, *FGFR2* amplification is reported to occur in 2–9% of patients. FGFR2 gene amplification is described as an independent poor prognostic factor in GC patients and is associated with higher pT and pN, lymphovascular invasion and distant metastasis. FGFR could also be a promising predictive marker; thus, different inhibitory molecules of the receptor are being tested [36].

MET

MET is a tyrosine kinase receptor (RKT) belonging to the family of hepatocyte growth factor receptors (HGFRs). It binds HGF/SF (hepatocyte growth factor/scatter factor) and plays a central role in the process of embryonic development, wound healing and organ regeneration. Autophosphorylation of MET leads to the activation of several downstream pathways (PI3K, Akt and RAS-MAPK) responsible for cancer

cell survival, proliferation, invasion and metastasis. It is overexpressed in approximately 50% of advanced gastric cancer cases. It can be considered a negative prognostic factor. In fact, MET gene overexpression is related to a poor prognosis, and it is associated with a more aggressive disease and a shorter OS and disease-free survival than MET-negative gastric cancers. Furthermore, MET can be considered a predictive biomarker. The monoclonal antibody rilotumumab can prevent binding of the MET receptor with its ligand HGF; this targeted therapy, in association with chemotherapy, improves the survival of patients [37].

VEGF

Vascular endothelial growth factor (VEGF) is one of the most important factors driving tumour angiogenesis. The VEGF family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PlGF). These proteins act through specific tyrosine kinase receptors (VEGFR1, VEGFR2 and VEGFR3), expressed primarily on endothelial cells. High levels of angiogenic factors in serum and tumours are associated with worse outcomes in patients with gastric carcinomas. In particular, VEGF-A, the most extensively studied angiogenic factor, could be a useful biomarker for disease progression and remission but not for diagnosis [38].

PIK3/mTOR

The phosphoinositide-3 kinase (PI3K)-protein kinase (PKB/AKT)-mammalian target of rapamycin (mTOR) pathway is a canonical pathway involved in anti-apoptosis and prosurvival and regulates several normal cellular activities, such as cell proliferation, survival and migration. Mutations in the PI3KCA gene, which encodes the alpha p110 catalytic subunit of PI3K, lead to constitutive activation of the PI3K/mTOR pathway. The PIK3CA mutation has been associated with a worse prognosis, reduced survival and increased lymph node metastasis. Therefore, it can be considered a negative prognostic factor [39].

In the last few years, targeted therapies have been tested to inhibit the PI3K/mTOR pathway, such as the one with everolimus, an mTOR inhibitor that seems to promise an improvement in survival in patients with gastric carcinoma. Thus, mTOR can probably also be used as a predictive marker [40].

Microsatellite Instability (MSI)

Microsatellite DNAs are widespread, short and repetitive DNA sequences that are randomly distributed in the human genome. When mismatch repair genes, including *hMLH1* and *hMSH2*, are inactivated, replication errors, such as insertions or deletions of bases within microsatellite regions, cannot be repaired. These phenomena are known as microsatellite instability (MSI). Alternatively, MSI can also be caused by epigenetic promoter methylation. Based on the frequency of mutation, MSI is categorized as low level (MSI-L), high level (MSI-H) and microsatellite stable (MSS). MSI has been used in the prognosis of numerous types of cancer. In gastric cancer, MSI is mainly related to hereditary type that occurs because of mutations during DNA replication. Patients with gastric cancer and MSI-H tend to be older and female and have a distally located tumour, with a well-differentiated adenocarcinoma type and in lower tumour stages [41].

The association between MSI-H and gastric cancer prognosis remains ambiguous. Certain studies support that MSI-H is associated with a good prognosis, while others are conflicting [42].

PLK1

Polo-like kinase 1 (PLK1), also known as serine/threonine-protein kinase 13 (STPK13), is a regulator protein of the cell cycle. Its aberrant expression is a driver of cancerous transformation and progression in various neoplasms, including GC.

Different studies have suggested that GC patients with a high expression of PLK1 had an inferior survival outcome [43].

High expression of PLK1 promotes GC cell metastasis rates and epithelial-mesenchymal transition by regulating the activation of the protein kinase B pathway [44].

Thus, an in-depth understanding of the molecular mechanism of PLK1 in tumour metastasis and in the inferior prognosis may lead to the discovery of new prognostic models. PLK1 could be a valid prognostic marker, and it could play a significant role in future clinical trials.

Predictive Tissue Biomarkers

HER2

HER2 (encoded by the proto-oncogene ERBB2 located on chromosome 17) is one of the four members of the human EGFR family (EGFR or HER1, HER2, HER3 and HER4) in the receptor tyrosine kinase (RTK) superfamily. It is located in the nucleus, and it is involved in the regulation of cell proliferation, differentiation, motility and apoptosis. Amplification of the ERBB2 gene results in the overexpression of HER2 protein, leading to cancer cell survival, growth and proliferation through the PI3K-AKT and MAPK pathways. HER2 overexpression, identified previously in breast cancer, has become a very important predictive biomarker in GC that allows clinicians to identify patients who would have a survival benefit from biological therapy.

Accurate identification of patient candidates for treatment with HER2-targeted therapy is now a fundamental step to optimize the therapeutic strategies in gastric carcinoma and breast cancer. Immunohistochemical (IHC) examination of the state of HER2 is recommended in all patients with advanced or metastatic carcinoma. This analysis allows the definition of the expression of HER2 at the membrane level, defined as 0 (negative), 1+ (negative), 2+ (equivocal) or 3+ (positive) (Figs. 5.16 and 5.17).

Tumours with IHC 3+ positivity are eligible for treatment with specific agents, whereas equivocal cases (IHC 2+) should be tested with in situ hybridization (ISH) techniques to evaluate the amplification status of HER2. Although both fluorescence in situ hybridization (FISH) and silver in situ hybridization (SISH) are approved by the European Medicines Agency to retest immunohistochemistry 2+ HER2 samples, it is widely accepted that SISH is a more suitable methodology than FISH for gastric cancer because it uses bright-field methodology and, therefore, can rapidly detect HER2-positive tumour foci within a heterogeneous sample [45].

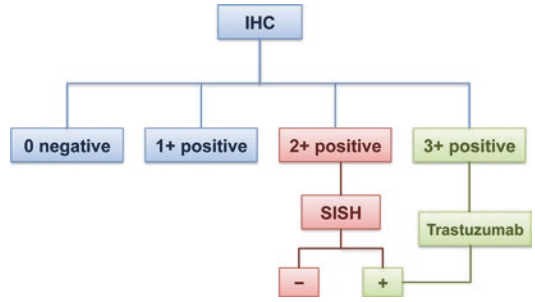

Fig. 5.16 Algorithm for the evaluation of HER2 in gastric carcinoma

The SISH "dual-colour" method, which uses two fluorochromes or two different chromogenes to visualize the same chromosome preparation on the same chromosome region 17 (CEP 17 probe) and the number of copies of the HER2 gene, is the most used and advisable approach (Fig. 5.18).

The definition of gene amplification is based on the evaluation of the relationship between HER2 gene signals and the centromeric signals of chromosome 17. The criteria for the definition are described in Fig. 5.19.

Currently, trastuzumab is the only targeted therapy permitted for advanced gastric cancer. Other drugs against HER2, such as lapatinib, pertuzumab and trastuzumab emtansine, are now being studied in clinical trials. Instead, the role of HER2 as a prognostic biomarker remains doubtful; indeed, some studies show an association of HER2 with a worse prognosis and a more aggressive disease, while others do not show a significant difference in the prognosis between HER2-positive and HER2-negative cancers. Some studies have suggested an association of ERBB2 amplification with tumour size, lymph node metastasis, local invasion and cancer stage; other studies have found no link between them [46, 47].

PD-1 and PD-L1

Programmed death 1 (PD-1) and its ligand programmed death ligand 1 and 2 (PD-L1 and

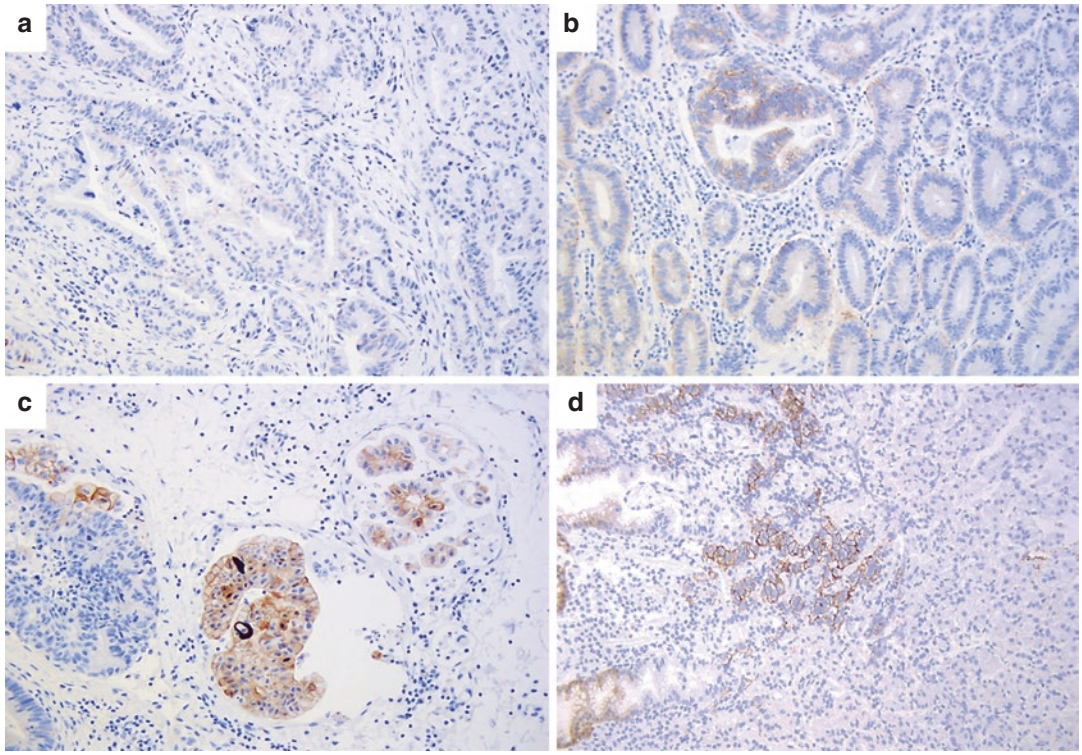

Fig. 5.17 (**a**) Immunohistochemical expression of HER2 (score 1+) in a gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain). (**b**) Immunohistochemical expression of HER2 (score 2+) in a gastric tubular-papillary adenocarcinoma original magnification 200× (haematoxylin counterstain). (**c**) Immunohistochemical expression of HER2 with vascular invasion in a gastric tubular-papillary adenocarcinoma original magnification 200× (haematoxylin counterstain). (**d**) Immunohistochemical expression of HER2 (score 3+) in gastric tubular-papillary adenocarcinoma, original magnification 200× (haematoxylin counterstain)

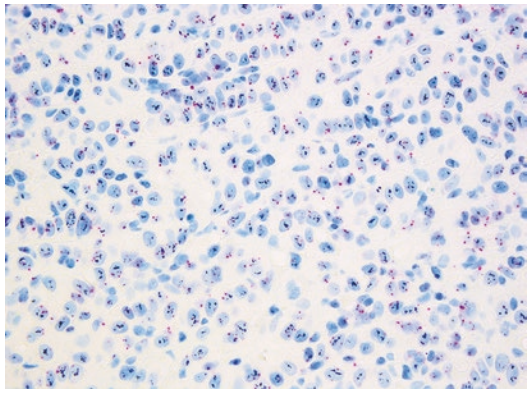

Fig. 5.18 Dual-colour SISH HER2 amplification in a gastric cancer, original magnification 200× (haematoxylin counterstain)

PD-L2) are a group of negative co-stimulatory molecules that can suppress T-cell proliferation in carcinoma. Neoplastic cells express the PD-1/PD-L1 pathway to escape the immune surveillance of T cells and the immune system response to the cancer [48].

The clinical efficacy of PD-1/PD-L1 inhibition has been observed for various malignancies, such as melanoma and non-small cell lung cancer (NSCLC), and it could be a promising way even in the treatment of gastric cancer. Types of cancers that show positive expression of PD-L1 are associated with a higher response rate to anti-PD-1/PD-L1 treatment. A monoclonal antibody anti-PD-1 pembrolizumab has manifested effi-

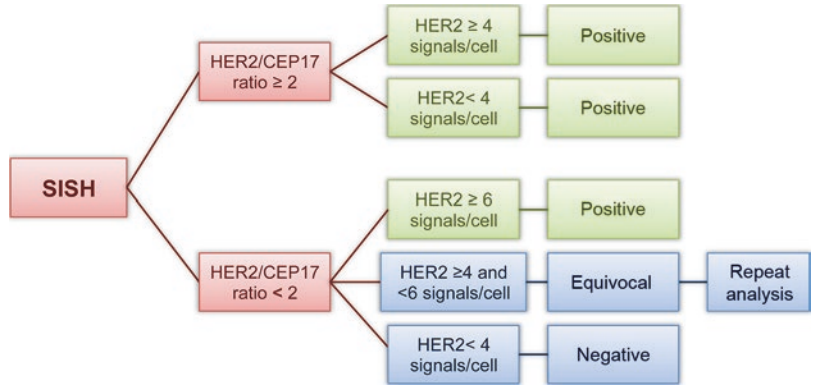

Fig. 5.19 Criteria for definition of HER2 amplification with SISH method

cacy in patients affected by advanced gastric cancer in the phase IB KEYNOTE-012 trial, a multicentre, open-label, phase 1b trial that included cohorts of patients with advanced gastric cancer and other cancer types [49]. Pembrolizumab is a selective, humanized, high-affinity IgG4-κ monoclonal antibody designed to bind to PD-1 and thus block the interaction between PD-1 and its ligand. It seems to have an acceptable safety profile and has shown promising antitumour activity in several types of advanced solid tumours [50].

Therefore, overexpression of PD-L1 can then be considered a predictive biomarker of the response to a targeted therapy. Targeting the PD1/PD-L1 pathway represents a promising strategy for the treatment of GC [51].

Correlation of GC and Tissue Biomarkers

Tissue Biomarkers in Precancerous Lesions

Immunohistochemistry and in situ hybridization may assist in the assessment of metaplasia and dysplasia. Most cases of GIM show CK7 and CK20 in the superficial and deep crypt cells [52] and are also positive for CDX2 [53].

The complete type of GIM is negative for MUC1, MUC5AC and MUC6 but positive for MUC2, while incomplete GIM is universally positive. *H. pylori* infection has been found in over 80% of patients with GIM and can then be identified using the Das-1 antibody, which stains *H. pylori* in gastric-associated GIM [54]. Interestingly, cells at the base of metaplastic glands are positive for LGR5+ and other intestinal stem cell (ISC) markers (OLFM4 and EPHB2). Because of the GI–I transition, this intestinal-like stem cell phenotype can be targeted to reverse IM and potentially prevent their progression into gastric cancers [55]. Otherwise, GEDs are often positive for p53 expression and Ki-67, and their expression increases according to the grading of dysplasia.

Tissue Biomarkers of GA

GA shows variable expression of CK7, CK20, CDX2, MUC2 and MUC5AC.

CK7 expression is an important marker of committed gastric epithelial cells and GA. Approximately 50% of GC are strongly positive for CK7. However, approximately 40% of GC show CK20 expression in a patchy or diffuse distribution. The positivity/negativity of both or one of the two markers seems to have the following percentages: approximately 35% of GC are CK7+ and CK20+, 25% are CK7− and CK20+, 25% are CK7+ and CK20–, and 15% are both CK7 and CK20−.

Several studies examined CDX2 in GC. Its expression seems to be variable and heterogeneous in the different types of GC.

Additionally, the various mucins in GC have different expression levels. GC can show the expression of MUC2 and MUC5AC, but it is almost always negative for MUC1 [56, 57].

Some variants of epithelial GA have been described. For example, recently, a new histological type of gastric adenocarcinoma called gastric adenocarcinoma with chief cell differentiation (GA-CCD) was described by Tsukamoto et al. [58]. Immunohistochemical evaluation of GA-CCD revealed diffuse positivity for MUC6 and negative staining for MUC5AC and MUC2 [59, 60].

As already stated, HER2 expression is evident in one out of five cases of stomach cancers often with a heterogeneous staining pattern [61].

Cases of poorly differentiated adenocarcinoma with prominent lymphoplasmacytic stroma may also be positive for the Epstein-Barr virus (EBV). Tumours of the upper gastrointestinal tract such as Barrett's oesophagus, oesophageal adenocarcinoma and gastric adenocarcinoma may show similar immunohistochemical findings.

In addition, it seems that 15–70% of common gastric carcinomas show expression of the neuroendocrine markers chromogranin A and synaptophysin.

In general, endocrine differentiation may be important for pathologic classification and could also be clinically relevant. In a particular study, the presence of endocrine differentiation detected with a cut-off of 20% of CgA+ and/or Syn+ tumour cells was significantly correlated with a higher relapse rate and higher disease-specific mortality than conventional tumours and tumours expressing CgA and/or Syn in 1–20% of cells [62].

A list of the most promising tissue biomarkers in gastric cancer is reported in Table 5.1.

Table 5.1 Emerging diagnostic, prognostic and predictive biomarkers and their targeted drug development

Biomarker characteristics	Diagnostic value	Prognostic value	Predictive value
Cytokeratins (*CK7; CK18; CK20; CDX2*): component of intermediate filaments involved in the fixation of the nucleus and maintenance of cell morphology	Important role for the diagnosis of GA	–	–
Mucin (*MUC1, MUC2 MUC5AC and MUC6*): mucosal protection and function	Has been used to evaluate the mucin phenotypes of gastric cancer	–	–
β-catenin: constituents of the cytoskeleton	Important role in the diagnosis of GA	–	–
Chromogranin A: neuroendocrine secretory protein	Diagnostic role in neuroendocrine carcinoma (NEC)	–	–
Synaptophysin: integral membrane glycoprotein	Diagnostic role in neuroendocrine carcinoma (NEC)	–	–
Cyclooxygenase 2 (*COX2*): conversion of arachidonic acid to prostaglandin H2	Useful in the diagnosis of GC	–	–
MLH1, MSH2, MSH6 and PMS2: detect and repair DNA mismatches that can occur during cell replication	Useful in the diagnosis of GC	–	–
P53: transcriptional factor whose function is to maintain genomic stability	Useful in the diagnosis of GC	Doubtful prognostic value	–
CEA: set of highly related glycoproteins that are involved in cell adhesion	–	It is associated with an increased risk of recurrence and a poor prognosis	–

(continued)

Table 5.1 (continued)

Biomarker characteristics	Diagnostic value	Prognostic value	Predictive value
E-cadherin: role in calcium-mediated adhesion and cell differentiation	–	It is associated with a worse prognosis and a lower survival rate	It is associated with a reduced response to conventional and targeted therapy
EGFR: tyrosine kinase receptors	–	It is associated with slightly differentiated to high-stage tumours and a low survival	–
FGFR: controls cellular proliferation and tumour growth, angiogenesis and dissemination	–	It is associated with a poor prognosis in GC patients	Probable predictive value
MET: role in the process of embryonic development, wound healing and organ regeneration	–	It is associated with more aggressive disease, a shorter OS and disease-free survival	It is a predictive biomarker of the response to rilotumumab
VEGF: the most important factors driving tumour angiogenesis	–	It is associated with worse outcomes in patients with gastric carcinomas	–
PIK3/mTOR: involved in anti-apoptosis and prosurvival	–	It is associated with a worse prognosis, reduced survival and increase lymph node metastasis	It is a predictive biomarker of the response to everolimus
Microsatellite instability (*MSI*): short and repetitive DNA sequences that are randomly distributed in the human genome	–	It is probably associated with well-differentiated adenocarcinoma	–
PLK1: is a multifaceted regulator of the cell cycle	–	It is associated with an inferior survival outcome	–
HER2: it is involved in cell proliferation, differentiation, motility and apoptosis	–	Still controversial prognostic value	It is a predictive biomarker of the response to trastuzumab
PD-1/ PD-L1: negative co-stimulatory molecules that can suppress T-cell proliferation in carcinoma	–	–	Overexpression is a predictive biomarker of the response to pembrolizumab

Promising New Biomarkers for Gastric Carcinoma

Matrix Metalloproteinase (MMP)

Matrix metalloproteinases (MMPs) are proteins that belong to a family of zinc-dependent endoproteinases, and their functions are to degrade elements of the extracellular matrix. They are involved in many physiological and pathological processes. MMPs are upregulated in gastric cancer, and they have been associated with specific disease characteristics of cancer. Different studies conducted on this topic require that MMP and TIMP, as well as their inhibitors, can be used as markers of invasion depth, peritoneal dissemination and metastasis. Unfortunately, currently, MMP inhibitors have not demonstrated a significant clinical benefit as therapy. However, their therapeutic role will be clarified in the future [63].

MicroRNA

MicroRNAs (miRNA) are 20- to 22-nucleotide noncoding RNA fragments whose functions are to bind the 3'UTR regions of their target genes and regulate their expression by modulating translation. MicroRNAs are involved in the regulation of different processes of the cell, such as proliferation, differentiation, migration and invasion [64]. It seems that they play a very important role in the carcinogenesis of gastric cancer; they can increase the expression of oncogenes or reduce the expression of tumour suppressor genes. Therefore, they may have prognostic significance and be associated with a worse prognosis.

Hepatocyte Nuclear Factor 4 Alpha (HNF4A)

HNF4A is a nuclear transcription factor that binds DNA as a homodimer. It is involved in different mechanisms such as invasion, metastasis and the epithelial-to-mesenchymal transition. Different studies have shown that HNF4A is a very good marker to discriminate between primary and metastatic gastric and breast carcinomas [65].

Other studies have suggested that HNF4α is a potential direct or indirect target for pharmacological drugs that act on the intestinal epithelium, and it seems that one HNF4α isoform was shown to correlate with Epstein-Barr viral infection in GC tumours [66].

FBXO2

FBXO2 belongs to the F-box family of proteins and is a cytoplasmic protein and ubiquitin ligase F-box protein with specificity for high-mannose glycoproteins. These proteins are classified into three different families based on the presence of specific recognition domains. Members of this family are important in cell cycle regulation, play key roles in tumourigenesis and have oncogenic or tumour-suppressive activities. A recent study has suggested that FBOX2 has clinical relevance in GC, and it demonstrates that FBXO2 levels are positively associated with lymph node metastasis, suggesting that high expression of FBXO2 could play an important role in the prediction of metastasis development in gastric cancer. Likely, the future role of FBXO2 in gastric cancer will provide promising new diagnostic biomarkers and therapeutic targets [67].

AURKA

Aurora kinase A (AURKA) is a protein belonging to a family of molecules that consists of highly conserved serine-threonine kinases that play critical roles in the regulation of mitotic events such as spindle assembly, function of centrosomes and cytoskeleton and cytokinesis. AURKA has been implicated in the regulation of cell cycle progression, mitosis and a key number of oncogenic signalling pathways in various malignancies. Several studies have shown that the overexpression of AURKA has been observed in early preneoplastic stages of gastric cancer in mouse models and humans [68].

Others have reported that AURKA promotes activation of the AKT prosurvival signalling pathway [69]. Furthermore, it was shown that AURKA can regulate and suppress GSK3β kinase activity in gastric cancer cell lines [70]. The interaction between AURKA and GSK3β causes the activation of β-catenin/TCF transcription complex, which leads to increased mRNA expression of different oncogenic proteins such as CCND1, c-MYC, c-MYC-binding protein, CLDN1, FGF18 and VEGF. However, little is known about its role in gastric cancer prognosis.

Thus, it seems that overexpression of AURKA mediates several pro-tumorigenic functions in addition to mitosis, thereby suggesting AURKA as a potential therapeutic target [71].

CDH17

Cadherin-17 (CDH17), which is also called liver-intestine (LI) cadherin, is a member of the cad-

herin superfamily of cell adhesion molecules, but its biological function remains unknown. CDH17 is expressed in mice and humans almost exclusively in the epithelial cells of both the embryonic and adult small intestines and colons, and it has no detectable expression in the liver or stomach [72]. Nevertheless, it seems that the protein is overexpressed in some GC cases. After the first study based on CDH17 as an intestinal metaplasia marker by Grotzinger et al. [73], several studies have evaluated CDH17 expression in gastric cancer. CDH17 was expressed in 50–78% of GC tissues with intestinal-type predominance [74, 75], suggesting that LI-cadherin is likely a marker for the intestinal phenotype [76].

REG4

Regenerating islet-derived family, member 4 (REG4) is a small secretory protein belonging to the group VII C-type lectin family, and its function may be related to proliferation and regeneration under physiological conditions [77].

Different studies have reported that REG4 is significantly overexpressed in GC tissues (especially in signet-ring cell carcinoma) compared with healthy tissue, and high expression of REG4 is positively related to lymph node metastasis [78].

Furthermore, it was reported that REG4 induces the expression of a series of anti-apoptosis genes (Bcl-2, Bcl-xl and survivin) by activating the epidermal growth factor receptor (EGFR)/protein kinase B (AKT)/activator protein 1 (AP-1) signalling pathway [79].

In the non-neoplastic stomach, foveolar epithelial cells do not express Reg 4, whereas goblet cells of intestinal metaplasia and neuroendocrine cells at the base of intestinal metaplasia express Reg 4, suggesting that Reg 4 is a marker for the intestinal phenotype [76, 80].

OLFM4

OLFM4 encodes olfactomedin 4 protein (also known as hGC-1 or GW112) and was originally cloned from human myeloblasts. Olfactomedin 4 expression is evident in crypt base columnar cells, which are intestinal stem cells in the intestinal metaplasia of the stomach; however, in the non-neoplastic stomach, it is not detected. Thus, this protein is useful to detect intestinal stem cells [81].

Some studies on well-differentiated adenocarcinomas have suggested that GC patients with positive expression of OLFM4 have a better survival rate than those with OLFM4-negative GC. The expression of olfactomedin 4 is frequently observed in the gastric phenotype so it likely plays an important role in the gastric phenotype of GC [82].

Both Reg4 and OLFM4 are secreted proteins, and serum Reg4 and OLFM4 serve as tumour markers for GC. The data suggest that serum OLFM4 combined with Reg4 is likely to be suitable for the screening of GC [76, 81].

HOXA10

Homeobox A10 (HOXA10) is a member of the homeobox gene family, which is evolutionarily well conserved and participates in several biological processes, such as the regulation of embryonic morphogenesis and differentiation and the control of normal development patterning along the anteroposterior axis [83].

It controls the organogenesis of the uterus during embryonic development and endometrial differentiation in adults. The deregulation of HOXA10 is correlated with the progression of endometrial carcinoma [84] but also promotes cell proliferation in other cancer types. The understanding of the role of HOXA10 in GC remains controversial, but HOXA10 appears to be expressed in the intestinal metaplasia of the stomach and may be a marker for the intestinal phenotype. Furthermore, the prognosis of patients with positive expression of HoxA10 is significantly better than that of patients with negative expression of HoxA10 [76, 85].

TSPAN8

TSPAN8 encodes tetraspanin 8 protein, which is a member of the tetraspanin family of proteins that span the membrane four times and is involved

in numerous biological processes [86]. In human cancers, overexpression of tetraspanin 8 has been shown to be related to hepatocellular carcinoma, pancreatic cancer, colon cancer and oesophageal cancer. It was also reported that TSPAN8 mRNA is upregulated in GC tissues compared to normal gastric tissues by microarray analysis. One study reported that TSPAN8 is not specific to gastric or intestinal GC but plays a crucial role in both phenotypes of GC [76].

Conclusion

Gastric cancer is a noteworthy disease due to its heterogeneous properties. Although the molecular alterations of this neoplasm have been under study for several years and despite the many advances in the diagnostic and prognostic fields, identifying new biomarkers and improving knowledge regarding known biomarkers are fundamental steps.

With the development of modern technologies, such as genome and exome sequencing and the use of miRNA microarrays, several new biomarkers have been identified with diagnostic, prognostic and predictive value.

Importantly, experimental trials are needed to screen new serum and tissue biomarkers before their clinical use and, thus far, promising results are expected in this field in the next future.

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Serum Biomarkers in Gastric Cancer

6

Agostino Steffan, Silvia Cervo, Valentina Fanotto, and Fabio Puglisi

Acronyms

AG Atrophic gastritis
AUC Area under the curve
CA 19-9 Carbohydrate antigen 19-9
CA 72-4 Carbohydrate antigen 72-4
CA 125 Carbohydrate antigen 125
CEA Carcinoembryonic antigen
cfDNA Cell-free DNA
CI Confidence interval
CTCs Circulating tumor cells
DSS Disease-specific survival
DFS Disease-free survival
G-17 Gastrin-17
GC Gastric cancer
HP *Helicobacter pylori*
HR Hazard ratio
IgG Immunoglobulin G
IM Intestinal metaplasia
NCA Nonspecific cross-reacting antigen
OR Odds ratio
OS Overall survival
PG Pepsinogen
TAG-72 Tumor-associated glycoprotein 72

Introduction

Circulating tumor biomarkers are defined as those substances produced by the tumor itself or by the organism in response to the presence of a neoplasia, which can be measured in the blood or in other biological fluids.

Serum tumor markers are blood-based biomarkers that are potentially useful in cancers detection, surveillance following curative surgery, prediction of drug response or resistance, and monitoring therapy in advance setting.

Irrespective of its application, the ideal tumor marker is represented by a biochemical indicator selectively secreted by cancer cells alone, which should theoretically allow an accurate and relatively

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_6

simple diagnosis of neoplasia; it should therefore exhibit a high positive and negative predictive value. Actually, the commonly used tumor biomarkers are neither specific nor sensitive; moreover, normal levels were set using the Gaussian function.

Another element of complexity in the interpretation of the clinical value of a given marker is certainly represented by the lack of homogeneity of the available data, which often derive from retrospective analyses with low statistical power. In order to standardize and increase the quality of the data reported in the studies that analyze potential prognostic factors in patients with cancer, the National Cancer Institute-European Organization for Research and Treatment of Cancer working group on cancer diagnostics, in 2005, set out specific methodological recommendations (Reporting Recommendations for Tumor Marker Prognostic Studies) [1]. However, the problem of the almost complete lack of prospectively validated data remains; this step would represent the confirmation of biological assumptions and retrospective analyses of many tumor markers.

Some tumor markers found in serum, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), can be elevated in 30–40% of gastric cancer (GC).

In addition to these commonly used markers, carbohydrate antigen 125 (CA 125) and carbohydrate antigen 72-4 (CA 72-4) have been reported to be elevated in advanced GC [2]. In 2010, the National Academy Clinical Biochemistry reiterated that no biomarker is recommended for routine clinical use during the diagnostic phase, while its use may be more useful in the course of the post-surgery follow-up and to monitor the response to antineoplastic treatment [3]. In line with these recommendations, international guidelines do not accept tumor markers in the process of GC diagnosis [4].

Their usefulness in GC can be acknowledged in:

- monitoring the effectiveness of antineoplastic therapy, but radiological assessment remains the gold standard [4];
- the surveillance period, but their role is controversial because an early detection of relapse does not necessarily translate into prognostic advantages [4].

The main characteristics of the classic tumor markers in GC are shown in Table 6.1.

Specific gastric biomarkers, i.e., pepsinogen (PG) I, PGII, gastrin-17 (G-17), and anti-*Helicobacter pylori* (HP) antibodies, are being used to identify patients at risk for development of GC, particularly combined in a panel test (GastroPanel) which provides comprehensive information on both the structure and the function of the entire stomach mucosa [5, 6].

CEA

CEA was initially isolated from fetal colon and colon cancer tissue in 1965 [7]; it is localized mainly to epithelial cell membranes facing the lumen in normal adult intestine, whereas it is found on adjacent cell membranes in both embryonic intestine and colon tumors [8]. CEA consists of a large family of related cell surface glycoproteins of which the major proteins are CEA and nonspecific cross-reacting antigen (NCA) [9]. Since the domain structures of CEA, NCA 50, and the heavy chain of immunoglobulin G (IgG) are very similar, CEA belongs to the immunoglobulin gene "superfamily". CEA is a glycoprotein with a molecular weight of 150 to 300 kDa; it is a single polypeptide chain consisting of 641 amino acids and containing 45–55% carbohydrate. It displays a cell adhesion activity and signal-regulatory properties [10].

CEA is normally present in the serum during the fetal period and then disappears in most adult individuals. The CEA concentration is elevated in a variety of cancers, such as colorectal (70%), lung (45%), gastric (50%), breast (40%), pancreatic (55%), ovarian (25%), and uterine (40%) carcinomas [11]. Therefore, it is not organ specific; in addition, its elevation could be associated with benign conditions, such as cirrhosis (45%), pulmonary emphysema (30%), rectal polyps (5%), benign breast disease (15%), and ulcerative colitis (15%) [11]. This, together with the number of tumors that do not produce CEA, does not recommend its testing for screening [12]. The half-life of CEA is 6–8 days; it has a hepatic metabolism [13]. Most assays use the immunometric format for determination of serum CEA; they use polyclonal

Table 6.1 Classic Tumor Markers in Gastric Cancer

	Chemical nature	Molecular weight	Half-life	Causes of elevated circulating level		
				Neoplastic	Benign	Other
CEA	Glycoprotein	200 kD	6–8 days	Colorectal carcinoma Potentially elevated in all types of adenocarcinoma	Chronic hepatopathy Cirrhosis Jaundice Pulmonary benign diseases Chronic renal failure Chronic respiratory diseases	Smoking Alcohol abuse
CA 19-9	Glycoprotein (mucin)	210 kD (purified form)	4–8 days	Pancreas and biliary tract carcinoma Other malignant tumors (stomach, colon, ovary, fallopian tubes, lung, breast, bladder)	Acute and chronic pancreatitis Cholelithiasis Jaundice Rheumatic and autoimmune diseases Diabetes Diabetic nephropathy Chronic hepatopathy Cirrhosis Acute hepatitis Benign pulmonary disease Cystic fibrosis	
CA 72.4	Glycoprotein (mucin)	220–400 kD	3–7 days	Gastric carcinoma Other malignant tumors: ovary, colon	Gastrointestinal diseases Heart disease Pancreatitis Hepatopathies Benign gynecologic disease (above all ovarian cysts) Renal failure Pneumonia Pulmonary fibrosis Rheumatic diseases Familial Mediterranean fever	Pregnancy Ibuprofen-treated pericardial effusion Steroids Omeprazole NSAIs

(continued)

Table 6.1 (continued)

	Chemical nature	Molecular weight	Half-life	Causes of elevated circulating level		
				Neoplastic	Benign	Other
CA 125	Glycoprotein (mucin)	500 kD	5–6 days	Ovary, fallopian tube, and endometrial carcinoma Other malignant tumors	Endometriosis Pelvic inflammation Hepatopathy Jaundice Leiomyoma of the uterus Systemic lupus erythematosus Inflammatory bowel diseases Serositis Congestive heart failure Pneumonia Pleurisy Acute pancreatitis Cirrhosis Acute hepatitis Autoimmune diseases Chronic renal failure Ascites Peritoneal inflammation	Pregnancy Menstruation Recent laparotomy Interferon

Analytic causes of variation of serum biomarker levels

Human antibodies directed against murine IgG used for the assay

False-positive mechanism: bridge between monoclonal antibodies; remedy: addition of murine serum or Ig

False-negative mechanism: steric interference with binding site; remedy: two incubation doses

and monoclonal antibodies or a combination of the two types. Normal values are of <3 ng/mL in nonsmokers or of <5 ng/mL in smokers. Since the concentration of CEA measured is method dependent, values should always be compared using the same method; if methods change, all patients who are being monitored should be tested in parallel using both old and new methods [11].

In 2015, a meta-analysis supported the association of elevated pretreatment serum CEA levels with a poor prognosis for GC patients in terms of overall survival (OS) [hazard ratio (HR) 1.716, 95% confidence interval (CI) 1.594–1.848)], disease-specific survival (DSS) (HR 1.940, 95% CI 1.563–2.408), and disease-free survival (DFS) (HR 2.275, 95% CI 1.836–2.818) [14]. The independent prognostic value of pretreatment serum CEA levels remains in patients with GC after adjustment for covariates (i.e., age, Borrmann type, CA 19-9, depth of invasion, sex, histology, liver metastasis, location, nodal involvement, TNM stage, tumor size, lymphatic invasion, and peritoneal metastases). Measurement during the postoperative follow-up would then be particularly important for those patients who had elevated preoperative values, although one cannot deny the relevance of measuring tumor markers among patients who did not have an elevated preoperative value [15]. In fact, more than 90% of patients with elevated preoperative levels of CEA had increased CEA levels again at the time of recurrence, whereas CEA levels increased for the first time at recurrence in 54.7% of cases; sensitivity for CEA for indicating recurrence was 65.8% [16].

Monitoring changing patterns in CEA to determine the relationship between changes in the serum levels and the response assessment in imaging studies throughout the treatment course revealed a significant correlation between the assessment of response by tumor markers and by imaging studies during systemic chemotherapy. However, although increases in serum tumor markers after chemotherapy were general indicators of tumor progression, an initial rise in the CEA levels after the start of chemotherapy should not be an indicator of progressive disease in some cases [15].

CA 19-9

Also known as the sialylated form of the Lewis A blood group antigen, this carbohydrate antigen is a glycolipid, denoted as Le^{xa}. Its expression requires the Lewis gene product, 1,4-fucosyl transferase; therefore patients who are genotypically $Le^{a\text{-}b\text{-}}$ (~5%) do not express CA 19-9 [11].

CA 19-9 is generally produced by normal human pancreatic and biliary ductal cells and by gastric, colon, endometrial, and salivary epithelia. Most of the CA 19-9 secreted by these cells is metabolized in serum, resulting in very low serum concentration in healthy people. On the contrary, in cases of pancreatobiliary carcinoma, epithelial cells of the tumor can produce a notable quantity of CA 19-9, resulting in an increased serum CA 19-9 level [17]. The antigen is found in serum as a mucin, which is a high molecular weight glycoprotein complex (200–1000 kDa).

The monoclonal antibody against CA 19-9 was developed from a human colon carcinoma cell line, SW-116 [18], and several immunoassays have been produced, with considerable differences among them, which make the results non-interchangeable for individual patients [19].

The CA 19-9 upper reference limit is 37 U/mL, as determined from the 99th percentile of normal subjects; this is the cutoff value able to discriminate between pancreatic cancer and benign pancreatic disease with clinical sensitivities of 69% and 93% and clinical specificities of 76–99% [11]. However, this value is often used for the diagnosis of GC, and it is unknown whether this cutoff is appropriate as a prognostic value [20]. CA 19-9 level >37 U/mL is found in patients with pancreatic (80%), hepatobiliary (67%), gastric (40–50%), hepatocellular (30–50%), colorectal (30%), breast (15%), and bladder cancers. However, 10–20% of patients with pancreatitis and other benign gastrointestinal conditions have elevated concentrations up to 120 U/mL [11].

In 2015, a meta-analysis including thirty-eight studies evaluated the relationship between CA 19-9 and clinicopathologic characteristics and the prognostic value of CA 19-9 in GC [20]. Results showed that there were significant differences in the incidence of high CA 19-9 levels according to stage (III/IV vs. I/II, odds ratio (OR) 3.36; 95% CI 2.34–4.84), pT classification (pT3/

T4 vs. pT1/T2, OR 2.40; 95% CI 1.60–3.59), nodal status (positive vs. negative, OR 2.91; 95% CI 2.21–3.84), distant metastases (yes vs. no, OR 2.76; 95% CI 1.12–6.82), and vascular invasion (yes vs. no, OR 1.66; 95% CI 1.11–2.48). In addition, CA 19-9 was significantly associated with poor OS (HR 1.83; 95% CI 1.56–2.15), DFS (HR 1.85; 95% CI 1.16–2.95), and DSS (HR 1.33; 95% CI 1.10–1.60) in GC.

Similar to CEA, dosing CA 19-9 during the postoperative follow-up seems to be particularly important for those patients who had elevated preoperative values; likewise, an initial rise in the CA 19-9 levels after the start of chemotherapy should not be an indicator of progressive disease in some cases [15].

CA 72-4

Initially called tumor-associated glycoprotein 72 (TAG-72), CA 72-4 is a mucin-like glycoprotein found on the surface of tumor cells, with a molecular weight of 200–420 kDa [2]. It was identified by the use of the monoclonal antibody B72.3, developed from the membrane-enriched fraction of breast carcinoma in a patient with liver metastases [11, 13]. CA 72-4 is not expressed by normal adult tissues, except for secretory phase endometrium and transitional mucosa of the colon; on the contrary, it could be expressed by tumors of epithelial origin (colorectal, gastric, ovarian, pancreatic, endometrial, and breast carcinomas) [13]. CA 72-4 is assayed in peripheral blood, but there are studies that compared serum levels to peritoneal lavage fluid levels [21]. Serum normal value (depending on the laboratory technique) is <6.9 U/mL, with a detection limit of 0.2 U/mL [22]. The overall sensitivity of this test is estimated to be of 40% in GC, with an overall specificity of 95% [23]. According to a review published in 2009, CA 72-4 is considered the major marker for GC, although it can also be associated with other carcinoma (e.g., colorectal cancer, pancreatic carcinoma, and lung cancer) [24]. In GC the positive rate of CA 72-4 is higher respect to those of CEA and CA 19-9 (30%, 21.1%, and 27.8%, respectively). Similarly to the other two biomarkers, CA 72-4 positive rate is higher in more advanced stages than in earlier ones. The positive rates for the three serum biomarkers were similar in detecting major tumors; however, CA 72-4 had the highest positive rate in patients with nodal involvement or serosal invasion. Therefore, CA 72-4 could be the most useful marker for detecting advanced GC [15]. In 2012, a meta-analysis of Chinese studies posed CA 72-4 as the most highly correlated serum tumor biomarker for GC in the Chinese population [25]. Elevated CA 72-4 was associated with tumor depth, nodal involvement, peritoneal and distant metastases, as well as stage [15, 26]. In a longitudinal study, Aloe and colleagues showed that the median presurgical serum CA 72-4 levels were significantly elevated in relapsing patients; moreover, positive presurgical serum CA 72-4 levels had an independent prognostic value in predicting recurrence [27].

CA 125

In 1981, Bast and colleagues identified the CA 125 glycoprotein antigen through the development of the OC 125 murine monoclonal antibody against cell line OVCA 433, which was developed from a patient with a serous papillary cystadenocarcinoma [28]. Subsequently, the CA 125 molecule has been cloned by the use of a partial cDNA sequence originating from the peptide core of the molecule identified. This new mucin molecule has been designated as CA 125/MUC16 [mucin 16, cell surface-associated (MUC16) gene] and consists of a 156-amino-acid tandem-repeat region in the N-terminus and a possible transmembrane region and tyrosine phosphorylation site in the C-terminus [29]. The first immunoassay for CA 125 used the OC 125 antibody for both capture and detection; afterward, a second-generation assay (CA 125 II) was developed, incorporating M11 and OC 125 antibodies, which have distinct nonoverlapping epitopes. Concentration of CA 125 may vary among manufacturers owing to differences in calibration, assay design, and reagent specificities. Values from different methods are not interchangeable,

so patients who are serially monitored should be reassessed if there is a change in methodology [30]. The cutoff of 35 U/mL for the CA 125 and CA 125 II assays was determined from the distribution of values in healthy population so as to include 99% of normal individuals [31]. As in other immunoassays, assay interferences may be observed if heterophilic antibodies are present in the serum, particularly following therapeutic or diagnostic use of monoclonal antibodies [30].

CA 125 is a differentiation antigen expressed by amniotic and coelomic epithelium during fetal development. In adults, it is found in the structures derived from coelomic epithelium (the mesothelial cells of the pleura, pericardium, and peritoneum) and in tubal, endometrial, and endocervical epithelium. The surface epithelium of normal fetal and adult ovaries does not express the determinant, except in inclusion cysts, area of metaplasia, and papillary excrescences [32].

Elevated serum CA 125 levels are associated with a variety of benign and malignant causes of pelvic mass; in fact, this marker could be increased in serum not only if gynecologic malignancies are present but also in endometriosis with endometriomas, salpingo-oophoritis with tubo-ovarian abscess, adenomyosis, leiomyomata uteri, and benign epithelial ovarian neoplasms [12]. In addition, increased CA 125 levels were found in conditions able to damage the peritoneal, pleural, and cardiac serosa, in renal failure, in hepatic and pulmonary diseases, as well as during pregnancy and during the menstrual cycle. Elevated levels of CA 125 were also observed in peritoneal carcinomatosis of ovarian or gastric origin [13]. Elevated serum CA 125 levels have been associated with peritoneal metastasis of GC [23, 33, 34].

Pepsinogen I and II

Pepsinogen (PG) is a precursor of the digestive enzyme pepsin. In humans, there are two isozymogens, PGI and PGII, with different biochemical and immunological properties [35, 36]. While PGI is synthesized by the oxyntic glands of the gastric mucosa, specifically by the chief cells and the mucous neck cells of the gastric corpus, PGII is also produced in the cardiac, pyloric, and duodenal Brunner gland cells [36]. Most of the PG is secreted into the gastric lumen, but a small amount (about 1%) can be found in the blood. Blood PG levels reflect the morphology and function of the gastric mucosa and other pathological conditions such as inflammation, HP infection, atrophic gastritis (AG), and intestinal metaplasia (IM) [37]. In the clinical practice, PGI levels and PGI/PGII ratio are often used for diagnosis; the ratio in normal subjects is about 4:1. Patients affected by AG are at increased risk of GC: the risk being even 5-fold in patients with advanced AG in the corpus and even 90-fold in advanced atrophic pangastritis (both corpus and antrum affected) compared to subjects with normal gastric mucosa [38].

The serum or plasma PGI assay seems to be a reliable test for detecting patients with advanced corpus AG [39]. PGI levels in the blood correlate with the number of chief cells in the gastric corpus mucosa. Since AG results in the loss of chief cells, it can be revealed by a linear decrease in blood PGI levels.

During the process of chronic AG, mucosal atrophy advances from the pyloric gland toward the oral side, and the PGI/PGII ratio decreases with the advancement of the disease [40]. The ratio is <3 when AG is advanced (moderate or severe) in the gastric corpus. The risk of GC is fivefold increased when PGI/PGII ratio is low [39].

The potential utility of PG as a diagnostic biomarker for AG and GC has been shown by numerous studies (it has been included in cancer screening programs in Japan as noninvasive test) [40]. In 2015 a meta-analysis, which included 31 studies involving 1520 GC patients and 2265 AG patients, identified a moderate capacity for serum PG to detect GC and AG [40]. The summary sensitivity and specificity for GC diagnosis were 0.69 (95% CI: 0.60–0.76) and 0.73 (95% CI: 0.62–0.82), respectively, with an area under the curve (AUC) of 0.76 (95% CI: 0.72–0.80). The summary sensitivity and specificity for AG diagnosis were 0.69 (95% CI: 0.55–0.80) and 0.88 (95% CI: 0.77–0.94),

respectively, with an AUC of 0.85 (95% CI: 0.82–0.88). It has to be noted that the between-study heterogeneity is dramatically marked, with sensitivity varying between 5.8% and 98.6% and specificity between 64.0% and 100%. Moreover, PG tests alone, which have been used for some time for screening of GC risk groups [41], have given only a modest impact on global GC mortality [42].

Gastrin-17

Gastrin is a gastrointestinal hormone which stimulates gastric acid secretion, promotes growth of gastrointestinal epithelial cells, and inhibits their apoptosis [43]. The gastrin gene is located on human chromosome 17q21 and encodes a 101-amino-acid polypeptide [44]. As a result of the post-translational maturation process of progastrin, the G-cells in the antrum release a mixture of acid stimulatory gastrins into the circulation. The dominant (80–90%) gastrin form in blood of healthy subjects is gastrin-17 (G-17), which is almost exclusively produced by the antrum G-cells [45], followed by gastrin-34 (5–10%). Fasting serum G-17 is considered as a noninvasive biomarker reflecting the structure and functional status of gastric mucosa. G-17 concentration, in fact, depends on intragastric acidity, on the number of antrum G-cells, and normally increases after food stimulation. Secretion of gastrin is regulated by pH level in gastric cavity: gastrin level decreases along with increase of gastric acid and considerably increases after eating [46]. In antrum AG, mucous antrum membrane is atrophied, and, as a consequence, the number of G-cells decreases, resulting in a reduction of G-17 secreted into circulation. Conversely, when gastric body is atrophied, gastric acid reduces, and then the secretion of G-17 increases together with its concentration in blood [47].

Since the 1990s [48], several studies of G-17 as a biomarker of GC screening have been conducted; anyway its clinical utility remains unclear, and the cutoff points for different gastric diseases are not established [49]. In 2016, two different meta-analyses evidenced that G-17 is able to detect AG with 48% sensitivity and 79% specificity [50], in particular AG of the antrum with 53.8% pooled sensitivity and 84.1% pooled specificity [5].

GastroPanel

GastroPanel test was designed by the Finn Biohit Oyj in the late 1990s to meet the increasing demand of noninvasive test for screening of GC risk. This ELISA-based panel includes three markers of mucosal atrophy (PGI and PGII for the corpus, G-17 for the antrum) and a HP IgG antibody assay. The added value of using this panel instead of the single biomarkers lies in the fact that it provides comprehensive information on both the structure and the function of the entire stomach mucosa, not restricted to either corpus or antrum alone [5, 6].

Since GC develops in a stepwise manner, subjects presenting precancerous lesions (AG, IM, and dysplasia) are at risk of developing carcinoma. HP, as a causative etiological agent of AG, is a well-established precursor of non-cardiac GC [51, 52]. It has been estimated that up to 1.8%, 10%, and 73% of patients affected by AG, IM, and dysplasia, respectively, progress to GC [53]. It is then crucial to identify the high-risk subjects in order to improve the GC diagnosis.

The rationale of GastroPanel test is based on the differential site of production of the biomarkers:

- PGI is only secreted by the corpus mucosa;
- PGII is also produced in the gastric antrum and duodenum;
- G-17 is only secreted by the antral mucosa;

and then: [6, 54]

- patients affected by corpus AG show lower blood PGI or PGI/PGII ratio;
- patients affected by antrum AG show low blood G-17 combined with positive anti-HP antibodies.

Even if GastroPanel has been designed for screening of patients at risk for GC and not for

invasive GC, it has been tested in different settings, also including first-degree relatives of GC patients and autoimmune chronic AG [55]; moreover, GC and gastric precancerous lesion occurrence show a significant geographic variation [5]. For this reason its sensitivity and specificity in AG and GC diagnosis show high variability among studies [5]. Moreover, studies from different geographic regions use different cutoff values for the single biomarkers, representing a critical source of bias [5].

In 2016, a meta-analysis covering all previous published studies on GastroPanel applied to AG diagnosis reported a better performance in diagnosing corpus AG (pooled sensitivity, 70.2%; pooled specificity, 93.9%) than antrum AG (pooled sensitivity, 53.8%; pooled specificity, 84.1%) [5]. A more recent meta-analysis (2017) found similar results for the diagnosis of corpus AG (sensitivity, 70.4%; specificity, 98.4%) but a higher performance for the diagnosis of antrum AG (sensitivity, 64.5%; specificity, 95.1%) [6]. Overall, the reported performance of the GastroPanel test for AG at any grade of severity and location is 74.7% sensitivity, 95–6% specificity, and 91% negative predictive value [6]. It has to be noted that, in both cases, most studies were conducted in Europe, so the test performance should be better explored in other populations.These findings would support the use of this test for diagnosis and screening of AG, to identify subjects to refer to endoscopy, as advocated by an international panel of expert [54]. Due to its high specificity, this assay represents an effective test for stomach health [5, 6, 54, 56] with high longitudinal negative predictive value for GC [49, 57].

Recent Advances and Future Perspectives

In the era of precision medicine, an extremely intriguing tool is represented by the so-called liquid biopsy, which can be generically considered a sample of any body fluid that may potentially contain material derived from a tumor. In the peripheral blood of cancer patients, it could be retrieved intact circulating tumor cells (CTCs), cell-derived vesicles (i.e., exosomes), and cell-free RNA and DNA (cfDNA) from the tumor. The most widely accepted hypothesis is that tumor cells release DNA in the bloodstream via apoptosis, necrosis, or cell secretion in tumor microenvironment [58].

Blood tests detecting somatic mutations provide high specificity as they search for driver gene mutations which are expected to be present only in abnormal clonal proliferations of cells [59–61]. Also in gastrointestinal cancers, liquid biopsy may represent a prognostic or predictive biomarker and a noninvasive tool for monitoring disease in terms of evaluation of response to systemic therapy as well as in monitoring clonal evolution [62]. Another purpose of liquid biopsy is that of screening and earlier detection. However, mutation-based liquid biopsy was principally used in patients with advanced cancers, since patients with early stage disease can harbor a plasma concentration of mutant template molecules which is often beyond the limit of detection of the most diffuse technologies [63]. Recently, a new multi-analyte blood test called CancerSEEK was developed; it combined eight protein biomarkers (CEA, CA 125, CA 19-9, prolactin, hepatocyte growth factor, osteopontin, myeloperoxidase, and tissue inhibitor of metalloproteinases 1) with genetic biomarkers, thus increasing sensitivity without decreasing specificity. Regarding patients with diagnosis of non-metastatic clinically detected GC, the sensitivity of CancerSEEK was almost 70%; moreover, this test, without any clinical information about the patients, was able to localize the source of the positive test to a single organ in a median of 63% of patients [64]. The combination of a multi-analyte test with other non-blood-based screening test could provide more information in order to get to an earlier detection of malignancy, which is crucial to reducing cancer deaths.

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Part III

Gastric Cancer Therapy: Multimodal Treatment Approach

7 New Agents in the Treatment of Advanced Gastric Cancer: Targeted Therapy and Immunotherapy

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Abbreviations

ASCO	American Society of Clinical Oncology
ATM	Ataxia telangiectasia
BSC	Best supportive care
CDH-1	Cadherin-1
CIN	Chromosomal instability
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
EGJ	Esophagogastric junction
ERK	Extracellular signal-regulated kinases
FGFR2	Fibroblast growth factor receptor 2
GATA6	GATA-binding protein 6
GC	Gastric cancer
GS	Genomically stable
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
JAK2	Janus kinase 2
KLF5	Kruppel-like factor 5
KRAS	Kirsten rat sarcoma viral oncogene homolog
MET	Mesenchymal-epithelial transition

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_7

MS	Microsatellite unstable
mTOR	Mechanistic target of rapamycin
OS	Overall survival
PARP	Poly-ADP ribose polymerase
PD-1	The programmed cell death 1 protein
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PFS	Progression-free survival
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
RHOA	Ras homolog gene family, member A
RR	Response rate
RTK	Receptor tyrosine kinase
TCGA	The Cancer Genome Atlas
T-DM1	Trastuzumab emtansine
TKI	Tyrosine kinase inhibitors
VEGFR	Vascular endothelial growth factor receptor

Introduction

Gastric cancer is the third leading cause of cancer mortality and the fifth most commonly diagnosed malignant disease [1]. The prognosis of GC is poor, especially for patients with metastatic disease, for whom the 5-year overall survival (OS) rate is approximately 5% [2]. For these patients, systemic therapy is the mainstay of treatment, and the goals of this therapy include palliation of symptoms and prolongation of survival. Systemic treatment with chemotherapy was the first to show a survival benefit over best supportive care (BSC) [3]. Despite some benefits from chemotherapy regimens, including docetaxel, fluoropyrimidines, irinotecan, cisplatin, and oxaliplatin, metastatic disease has a dismal prognosis, with a median OS of approximately 11 months for patients not harboring human epidermal growth factor receptor 2 (HER2) overexpression [4]. Conventional cytotoxic chemotherapy has been the backbone of advanced gastric cancer (GC) treatment for decades and still represents a key element of the therapeutic armamentarium. However, only small increments in survival outcomes have been reached.

For locally advanced GC, perioperative chemotherapy in the West and adjuvant chemotherapy in the East are standard. In stage IV chemotherapy prolongs survival and controls cancer-related symptoms [5]. Oxaliplatin and cisplatin plus fluoropyrimidine (5-fluorouracil, capecitabine, or S-1) are standard in a first-line setting. The addition of a third drug increases response rates and survival outcomes but leads to significant increases in toxicity [6, 7]. Patients for triplet chemotherapy should therefore be selected carefully. Responses to chemotherapy are often of short duration, and median overall survival (OS) in advanced GC is no longer than 8–11 months median in the West and 13–17 months in East Asia/Japan.

Over the past several decades, we have witnessed the advent of precision medicine, and remarkable advancements in the fields of targeted therapy and immunotherapy have recently been achieved. Precision medicine involves characterizing the molecular pathways of carcinogenesis and pharmaceutical development of monoclonal antibodies and small molecule inhibitors that interfere with crucial molecular targets.

A better understanding of genetic alterations and molecular signatures of gastric cancer has been reached in the last years. It will serve as a roadmap for better treatment stratification and future drug development. GC has numerous somatic genetic alterations, some of them contributing to chemotherapeutic resistance [8].

The molecular characterization of GC is rapidly evolving. Genes related to RTK/RAS signaling, in particular FGFR2, KRAS, ERBB2, EGFR, and MET, can be amplified in GC. These amplifications are frequently but not universally mutually exclusive [9, 10].

The Cancer Genome Atlas (TCGA) Project (Table 7.1) performed a comprehensive molecular evaluation of 295 gastric adenocarcinomas and has proposed a molecular classification scheme by which GC is categorized into four subtypes: Epstein-Barr virus (EBV)-positive tumors, microsatellite unstable (MSI) tumors, genomically stable (GS) tumors, and tumors with chromosomal instability (CIN) [11]. EBV-positive tumors represent 9% of gastric adenocarcinomas and display recurrent phosphatidylinositol 3-kinase CA

Table 7.1 The new molecularly based classification of GC according to the Cancer Genome Atlas (TCGA) 2014

Subtype	Epstein-Barr virus (EBV)-infected tumors	Microsatellite instability (MSI) tumors	Genomically stable (GS) tumors	Tumors with chromosomal instability (CIN)
Typical molecular features	EBV positive Profound hypermethylation CDKN2A silencing 80% PIK3CA mutation PD-L1/PD-L2 overexpression	DNA hypermethylation Silencing of MLH1 Elevated somatic mutations (PIK3CA 42% and ERBB3 26%)	Tumors lacking aneuploidy and elevated rates of mutation or hypermethylation Somatic RHOA and CDH-1 mutations CLDN18-ARHGAP6 or ARHGAP26 fusions	Marked aneuploidy TP53 mutations Recurrent amplifications of receptor tyrosine kinases (HER2 24%)
Association with anatomy or traditional subtypes	Fundus and body	Fundus, body, and antrum	Mostly diffuse subtype	Majority of tumors at the esophagogastric junction

(PIK3CA) mutations and amplification of HER2, JAK2, and programmed cell death-ligands 1 and 2 (PD-L1 and PD-L2). The MSI subtype represents 22% of GCs and is prevalent in women and older adults. These tumors are strongly associated with MLH1 promoter hypermethylation and show elevated mutation rates, elevated levels of microsatellite instability, and recurrent mutations in PIK3CA, HER3, and HER2. GS tumors are observed in 20% of GC patients, are enriched for the diffuse-type adenocarcinoma, and have frequent mutations in RHOA and CDH-1. Fusions involving RHO-family GTPase-activating proteins (CLDN18 and ARHGAP26) are also enriched in this subtype, and their fusion products impact RHOA function, which is involved in cell contractility and cellular motility. Finally, the CIN subtype accounts for 50% of gastric adenocarcinomas, is enriched by intestinal histology, and shows frequent TP53 mutations and receptor tyrosine kinase (RTK)/RAS amplifications [11, 12].

Another notable study sought to identify the most prevalent molecular alterations in GC. The authors identified 22 recurrent focal somatic copy number alterations including known targets such as fibroblast growth factor receptors 2 (FGFR2) and HER2 and also novel genes such as KLF5 and GATA6. Interestingly, RTK/RAS amplifications were frequent and occurred in approximately 37% of GCs, and KRAS amplifications were also frequent and associated with an adverse prognosis [9]. The design of future GC trials, particularly in molecularly targeted and immune therapy, should consider genetic and immunity differences, as they may impact treatment response and clinical outcomes.

Targeted Agents

Data from systematic profiling studies has revealed numerous molecular alterations in GC. This increased knowledge has significantly improved pharmaceutical development to design and clinically test selective inhibitors against proteins and lipid kinases that play crucial roles in carcinogenesis.

Anti-HER2 Agents

HER2 is a tyrosine kinase member of the epidermal growth factor receptor (EGFR) family. HER2 is involved in the carcinogenesis of many types of cancer, and its overexpression can be identified in up to 30% of GCs with some differences regarding histological and location characteristics. The overexpression is more common in the intestinal type (34%) than in the diffuse type (6%) and more prevalent in esophagogastric junction (GEJ) tumors (32%) than other locations of the stomach (18%) [13, 14].

Trastuzumab, a recombinant humanized monoclonal antibody against HER2, was the first targeted agent to be approved for GC in 2010. The approval was based on a phase 3 trial (ToGA) that evaluated 594 patients with HER2-positive advanced gastric or EGJ cancer. Trastuzumab (8 mg/kg loading dose, then 6 mg/kg every 3 weeks) was investigated as a first-line treatment in association with chemotherapy consisting of capecitabine plus cisplatin or fluorouracil plus cisplatin administered every 3 weeks for six cycles. The median OS was 13.8 months for the trastuzumab plus chemotherapy arm and 11.1 months for patients in the chemotherapy-alone arm (hazard ratio (HR) 0.74; 95% CI 0.60–0.91; $P = 0.0046$). The response rate (RR) was also higher in the experimental arm (47% versus 35%), as was the median progression-free survival (PFS) (6.7 months versus 5.5 months; HR 0.71; $P = 0.0002$) [14].

Other HER2 blockade drugs were not as successful as trastuzumab. The phase 3 LOGIC trial evaluated the efficacy of lapatinib, a tyrosine kinase inhibitor of EGFR and HER2, as a first-line treatment in combination with chemotherapy (capecitabine plus oxaliplatin). The median OS of the experimental arm was not significantly different from that of the control arm of chemotherapy alone (12.2 versus 10.5 months; HR 0.91; $P = 0.3492$) (check Table 7.2 for details) [15]. The TYTAN trial evaluated lapatinib in the second-line setting with paclitaxel. Similar to the LOGIC trial, the median OS was not significantly different (11.0 months for lapatinib and paclitaxel versus 8.9 months for paclitaxel alone; $P = 0.1044$) [16] (Table 7.2). Trastuzumab emtansine (T-DM1) also failed to show survival advantage over standard chemotherapy. The phase 3 GATSBY trial investigated the efficacy of T-DM1 in patients previously treated for HER2-positive GCs. The median OS was 7.9 months with T-DM1 and 8.6 months with taxane (HR 1.15; one-sided $P = 0.86$) [17] (Table 7.2). Currently, the phase 3 JACOB trial (NCT01774786) is ongoing and will evaluate the efficacy and safety of pertuzumab in combination with trastuzumab, fluoropyrimidine, and cisplatin as a first-line treatment in participants with HER2-positive metastatic GCs.

Anti-vascular Endothelial Growth Factor Receptor (VEGF) Agents

Ramucirumab, a recombinant monoclonal antibody that binds to VEGFR-2, is approved alone and in combination with paclitaxel as a second-line treatment based on two randomized phase 3 trials. The REGARD trial randomized 355 patients, who showed disease progression during first-line platinum-containing or fluoropyrimidine-containing treatment to ramucirumab alone (8 mg/kg IV every 2 weeks) or placebo. The median OS was 5.2 months for the ramucirumab arm and 3.8 months for the placebo arm (HR 0.776; $P = 0.047$). Median progression-free survival was 2.1 months in patients receiving ramucirumab and 1.3 months in those receiving placebo (HR 0.483; $P < 0.0001$). The RR was 3% in both arms [18]. The RAINBOW study compared weekly paclitaxel (80 mg/m^2 on days 1, 8, and 15 of each 28-day cycle) plus ramucirumab (8 mg/kg IV every 2 weeks) to a placebo arm using 665 patients with metastatic GC or EGJ cancer after first-line platinum- and fluoropyrimidine-based combination therapy. The median OS was significantly longer in the ramucirumab arm versus that in the placebo arm (9.6 months versus 7.4 months; HR 0.807; $P = 0.017$) as well as the median PFS (4.4 months versus 2.9 months; HR 0.635; $P < 0.0001$). The RR was also greater in the ramucirumab plus paclitaxel arm (28% versus 16%; $P = 0.0001$) [19].

The benefit of bevacizumab, a monoclonal antibody that binds to soluble VEGF and prevents binding to VEGFR, is uncertain. The AVAGAST trial investigated bevacizumab as a first-line treatment with capecitabine plus cisplatin every 21 days for a maximum of six cycles. Thereafter, capecitabine plus either bevacizumab or placebo was continued until disease progression. There was no significant survival benefit for the experimental arm over the control arm (median OS of 12.1 versus 10.1 months, HR 0.87; $P = 0.1002$), but the median PFS (6.7 versus 5.3 months; HR 0.80; $P = 0.0037$) and overall RR (46.0% versus 37.4%; $P = 0.0315$) were significantly improved [20] (Table 7.2). The AVATAR

Table 7.2 Gastric cancer-targeted therapy—negative trials

Study	Phase	Line	No.	Investigational arm	Control arm	RR	PFS	OS
LOGIC	3	First	545	Lapatinib + capecitabine and oxaliplatin	Capecitabine + oxaliplatin	53% vs 39%; $P = 0.0031$	6.0 vs 5.4 months; $P = 0.0381$	12.2 vs 10.5 months; $P = 0.3492$
TYTAN	3	Second	261	Lapatinib + paclitaxel	Paclitaxel	27% vs 9%; $P < 0.001$	5.5 vs 4.4 months; $P = 0.244$	11.0 vs 8.9 months; $P = 0.1044$
GATSBY	3	Second	345	T-DM1	Taxane	20.6% vs 19.6%; $P = 0.8406$	2.7 vs 2.9 months; $P = 0.31$	7.9 vs 8.6 months; $p = 0.86$
AVAGAST	3	First	774	Bevacizumab + capecitabine+ cisplatin	Capecitabine + cisplatin	46.0% vs 37.4%; $P = 0.0315$	6.7 vs 5.3 months; $P = 0.0037$	12.1 vs 10.1 months; $P = 0.1002$
AVATAR	3	First	202	Bevacizumab + capecitabine+ cisplatin	Capecitabine + cisplatin	41% vs 34%; $P = 0.35$	6.3 vs 6.0 months; $P = 0.47$	10.5 vs 11.4 months; $P = 0.56$
Lee et al.	2	Second	107	Sunitinib + docetaxel	Docetaxel	41.1% vs 14.3%; $P = 0.002$	3.9 vs 2.6 months; $P = 0.206$	8.0 vs 6.6 months; $P = 0.802$
EXPAND	3	First	904	Cetuximab + capecitabine + cisplatin	Capecitabine + cisplatin	30% vs 29%; $P = 0.77$	4.4 vs 5.6 months; $P = 0.32$	9.4 vs 10.7 months; $P = 0.95$
REAL3	3	First	553	Panitumumab + epirubicin, oxaliplatin, and capecitabine	Epirubicin + oxaliplatin+ capecitabine	46% vs 42%; $P = 0.42$	6.0 vs 7.4 months; $P = 0.068$	8.8 vs 11.3 months; $P = 0.013$
GRANITE-1	3	Second or third	656	Everolimus	Placebo	4.5% vs 2.1%	1.7 vs 1.4 months; $P < 0.001$	5.4 vs 4.3 months; $P = 0.124$

trial was a phase 3 study, similar to the AVAGAST trial, which was conducted only in Chinese patients. Similar to AVAGAST, the AVATAR trial showed that, compared with the placebo plus chemotherapy, addition of bevacizumab to capecitabine-cisplatin chemotherapy did not improve the median OS (10.5 versus 11.4 months, HR 1.11; $P = 0.56$) [21] (Table 7.2).

Apatinib, an orally active VEGFR-2 inhibitor, was evaluated in a phase 3 Chinese trial that randomized 267 patients with advanced GC or EGJ adenocarcinoma who had progressed through two or more prior lines of chemotherapy. Patients received 850 mg oral apatinib or placebo once daily. The median OS was modestly, but significantly, prolonged (6.5 versus 4.7 months; HR 0.709; $P = 0.0156$), and the median PFS was also improved (2.6 versus 1.8 months; HR 0.444; $P < 0.001$) [22]. Apatinib is approved in China for treatment of advanced GC but is not available in the United States or Europe.

Sunitinib and sorafenib are tyrosine kinase inhibitors (TKIs) that inhibit VEGFR-1, VEGFR-2, and VEGFR-3, as well as other tyrosine kinases. Sunitinib was investigated in a randomized phase 2 trial as a second-line therapy in combination with docetaxel. The primary time-to-progression endpoint was not significantly prolonged with the combination therapy compared with docetaxel alone (3.9 months versus 2.6 months, HR 0.77; $P = 0.206$) [23] (Table 7.2). Sorafenib was evaluated in a phase 2 trial in combination with docetaxel and cisplatin as a first-line treatment for metastatic GC or EGJ adenocarcinoma. The median OS was 13.6 months, the median PFS was 5.8 months, and the objective RR was noted in 41% of patients [24].

Anti-EGFR Agents

EGFR overexpression occurs in 2.3–40% of GCs, depending on the study and the methodology used to investigate the overexpression (immunohistochemistry or fluorescence in situ hybridization) [10]. However, targeted agents against EGFR have had disappointing clinical outcomes. The phase 3 EXPAND trial evaluated cetuximab, a chimeric monoclonal antibody against EGFR, in a first-line setting with chemotherapy (capecitabine and cisplatin). The median PFS (primary endpoint) was 4.4 months for chemotherapy plus cetuximab and 5.6 months for patients in the chemotherapy-alone arm (HR 1.09; $P = 0.32$) [25] (Table 7.2). Similarly, the REAL3 trial enrolled patients in a first-line setting for chemotherapy (epirubicin, oxaliplatin, and capecitabine) with or without panitumumab (a fully human monoclonal antibody against EGFR). The median OS, which was the primary endpoint, was 8.8 months for chemotherapy plus panitumumab versus 11.3 months for the chemotherapy-alone arm (HR 1.37; 95%; $P = 0.013$) [26] (Table 7.2).

PI3K/AKT/mTOR Pathway Inhibition

PI3K/AKT/mTOR is one of the most frequently activated pathways in human cancer and is activated in up to 60% of GCs [27]. Everolimus, a mechanistic (formerly known as mammalian) target of rapamycin (mTOR) inhibitor, was investigated in a phase 3 trial (GRANITE-1) in which 656 patients were randomized to the everolimus (10 mg daily) or placebo group after progression to one or two lines of systemic chemotherapy. The median OS was not significantly different (5.4 months for the everolimus arm versus 4.3 months for the placebo arm, HR 0.90; $P = 0.124$), and the median PFS was modestly improved (1.7 months for the everolimus arm versus 1.4 months for the placebo arm, HR 0.66; $P < 0.001$) [28] (Table 7.2). Currently, another phase 3 trial is investigating everolimus in a second-line setting in association with paclitaxel (NCT01248403).

Several other drugs that target the PI3K/AKT/mTOR pathway are under investigation. AZD5363, an AKT inhibitor, is being investigated in two phase 2 trials in combination with paclitaxel as a second-line treatment for patients with GC harboring a PIK3CA mutation (NCT02451956) and in biomarker-negative (PIK3CA/MEK/RAS/TP53/MET) patients

(NCT02449655). Another randomized phase 2 trial is investigating the efficacy of GDC-0068, another AKT inhibitor, in combination with modified FOLFOX6 in a first-line scenario (NCT01896531). Finally, a phase IB dose-escalation study is evaluating the PI3K inhibitor BYL719 in patients with GCs harboring a PIK3CA mutation or HER2 amplification (NCT01613950).

c-MET Inhibitors

Mesenchymal-epithelial transition (MET) receptor amplification or overexpression occurs in 0–23% of GCs [29]. c-MET inhibitors have been tested in GC patients with disappointing results. Two phase 3 trials investigated the safety and efficacy of rilotumumab, a monoclonal antibody against c-Met. RILOMET-1 and RILOMET-2 were designed to test rilotumumab in combination with chemotherapy as a first-line treatment. Both trials were closed in November 2014 based on an increase in the number of deaths in the rilotumumab and chemotherapy arms [29]. METGastric was another phase 3 trial that evaluated onartuzumab, a monovalent anti-MET antibody; enrollment was halted early due to the negative results in a phase 2 trial. The analysis of the 592 patients enrolled failed to show the benefit of onartuzumab associated with mFOLFOX6 in the first-line scenario [8]. Foretinib and tivantinib, TKIs against c-MET, also failed to show sustained activity in GC patients in phase 2 trials [11].

Fibroblast Growth Factor Receptor Blockade

Fibroblast growth factor receptors (FGFR1–FGFR4) are transmembrane tyrosine kinase receptors that play important roles in carcinogenesis by regulating angiogenesis and cell proliferation, migration, and differentiation. FGFR2 amplification is evident in approximately 5–10% of GC tumors and is associated with a poor prognosis [30, 31].

AZD4547 is a selective FGFR1–FGFR3 inhibitor that has been evaluated in comparison with paclitaxel in a randomized phase 2 trial (the SHINE study) as a second-line treatment for GC patients with FGFR2 polysomy or gene amplification. The PFS analysis did not show any statistically significant differences between the two arms [32]. Dovitinib is an oral multi-targeted TKI that targets FGFR1–FGFR3. A phase 2 trial is ongoing and evaluating dovitinib monotherapy as a salvage treatment in patients with metastatic GC harboring FGFR2 amplifications (NCT01719549). Another phase I/II study is evaluating dovitinib in association with docetaxel as a second-line treatment (NCT01921673).

Poly-ADP Ribose Polymerase (PARP) Inhibition

PARP, together with the ataxia telangiectasia (ATM) protein, plays an essential role in the DNA damage response [33]. Low ATM protein expression is evident in approximately 13–22% of tumors from patients with GC and is correlated with sensitivity to PARP inhibition [33, 34]. Olaparib is a PARP inhibitor that was investigated in a randomized phase 2 trial in which olaparib plus paclitaxel was compared with paclitaxel alone in a population of recurrent or metastatic GC patients whose disease had progressed after first-line chemotherapy; the population was enriched with patients with low or undetectable ATM levels. A total of 124 patients were enrolled, and the median PFS (primary endpoint) was not significantly different between the two arms (3.91 months for olaparib and paclitaxel arm and 3.55 months for paclitaxel alone arm; $P = 0.131$). However, the median OS was significantly improved in the overall population of the study in favor of the combination arm (13.1 versus 8.3 months, HR 0.56; $P = 0.005$), and the results were even more pronounced in the population with low ATM levels (not reached versus 8.2 months, HR 0.35; $P = 0.002$) [35]. A phase 3 trial is ongoing to evaluate this combination in the second-line setting (NCT01924533).

Claudin-18.2

Claudins constitute a family of proteins that participate in controlling the flow of molecules between cellular tight junctions. Isoform 2 of the tight junction molecule claudin-18 (CLDN18.2) is frequently expressed in GCs and is involved in carcinogenesis [36]. Claudiximab is a chimeric monoclonal antibody against CLDN18.2 [37]. The FAST trial, a phase 2b trial, evaluated the role of claudiximab in association with chemotherapy in the first-line scenario. A total of 161 patients with GC and EGJ tumors who were claudin-18.2 positive by immunohistochemistry were randomized to receive the EOX regimen (epirubicin 50 mg/m^2, oxaliplatin 130 mg/m^2 d1, and capecitabine 625 mg/m^2 bid, d1–d21, every 21 days) with or without claudiximab (loading dose 800 mg/m^2, then 600 mg/m^2 d1, every 21 days). The study met its primary endpoint with a median PFS of 7.9 months for the experimental arm versus 4.8 months for the chemotherapy-alone arm (HR 0.47; $P = 0.0001$). The median OS was also significantly higher for the claudiximab arm (13.3 versus 8.4 months; HR 0.51; $P < 0.001$) [37]. Future phase 3 trials evaluating the role claudiximab for GC patients are expected.

Immunotherapy Agents

Immunotherapy is already a reality in oncology and has achieved outstanding results in many cancer types [38–40]. The mechanisms involved in the immune suppression by the tumor are complex. The programmed cell death 1 protein (PD-1) and its ligands (PD-L1 and PD-L2) are key factors that control the ability of tumors to evade the immune surveillance [41]. Similarly, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) negatively regulates T-cell effector responses and is implicated in tumor immunological evasion signature [42]. Currently, several immunotherapy agents that address this mechanism are being tested as treatments for GC patients.

Pembrolizumab

Pembrolizumab is an anti-PD-1 monoclonal antibody. The phase 1b KEYNOTE 012 trial has evaluated 39 patients with PD-L1-positive gastric or EGJ tumors who received pembrolizumab (10 mg/kg every 2 weeks). This trial has shown manageable toxicities and promising results with 22% of patients achieving an overall response [43]. Early results of the KEYNOTE-059 trial were presented at the 2017 annual meeting of the American Society of Clinical Oncology (ASCO). Cohort 1 comprised 259 patients (not selected by PD-L1 status) who had progressed on ≥2 prior chemotherapy regimens and received pembrolizumab 200 mg every 3 weeks. The RR was 11.2% in the entire cohort and 15.5% for patients with PD-L1-positive tumors. Grade 3–5 treatment-related adverse events (AEs) occurred in 17% of patients [44]. In cohort 2, the safety and efficacy of pembrolizumab (200 mg every 3 weeks) plus chemotherapy (cisplatin 80 mg/m^2 + 5-FU 800 mg/m^2 or capecitabine 1000 mg/m^2 every 3 weeks) as a first-line treatment was evaluated. A total of 25 patients were enrolled with an RR of 60%, a median PFS of 6.6 months, and a median OS of 13.8 months. Grade 3–4 treatment-related AEs occurred in 76% of patients in this cohort [45]. Future trials will further clarify the role of pembrolizumab in the treatment of metastatic GC patients. The ongoing phase 3 KEYNOTE-061 trial is evaluating pembrolizumab versus paclitaxel as a second-line treatment (NCT02370498), and the phase 3 KEYNOTE-062 is evaluating pembrolizumab associated with cisplatin plus 5-FU as a first-line treatment (NCT02494583).

Nivolumab

Nivolumab is another anti-PD-1 monoclonal antibody with promising results in GC. The phase 1/2 CheckMate 032 study evaluated nivolumab with or without ipilimumab in heavily pretreated patients with gastric, esophageal, or EGJ cancers. Updated results were presented at the 2017 ASCO Annual Meeting. The study evaluated three cohorts: 59 patients received

3 mg/kg nivolumab every 2 weeks, 49 patients received 1 mg/kg nivolumab plus 3 mg/kg ipilimumab every 3 weeks (N1 + I3), and 52 patients received 3 mg/kg nivolumab plus 1 mg/kg ipilimumab (N3 + I1). In the nivolumab-alone cohort, the RR was 12%, and the median OS was 6.2 months [46].

The results from a phase 3 trial that evaluated nivolumab as a salvage treatment in 493 patients with gastric and EGJ cancers were presented at the 2017 ASCO Gastrointestinal Cancer Symposium. All patients had failed two or more previous chemotherapy regimens and were randomized to receive nivolumab 3 mg/kg or placebo every 2 weeks. The median OS was 5.32 months for the nivolumab arm versus 4.14 months for the placebo arm (HR 0.63; $P < 0.0001$). The RR was also significantly better for the nivolumab arm (11.2% versus 0%; $P < 0.0001$), as was the median PFS (1.61 months versus 1.45 months, HR 0.60; $P < 0.0001$). Grade 3 or higher treatment-related AEs occurred in 11.5% of patients in the nivolumab arm [47].

Ipilimumab

Ipilimumab is a monoclonal antibody that targets CTLA-4. A phase 2 study evaluated the safety and efficacy of ipilimumab versus BSC for patients with advanced gastric or EGJ cancers as a second-line treatment. Fifty-seven patients were randomized to 10 mg/kg ipilimumab every 3 weeks for four doses versus BSC. Immune-related PFS, the primary endpoint, was not improved (2.92 months for ipilimumab versus 4.90 months for BSC, HR 1.44; $P = 0.09$) [48].

As described above, the CheckMate 032 trial investigated the efficacy of nivolumab plus ipilimumab. The RR was 24% in the N1 + I3 cohort and 8% in the N3 + I1 cohort. The median OS was 6.9 months for the N1 + I3 patients and 4.8 months for the N3 + I1 patients. Grade 3–4 treatment-related AEs were higher for the N1 + I3 cohort than those for the nivolumab-alone patients and N3 + I1 patients. For example, grade 3–4 diarrhea was observed in 14% of patients in the N1 + I3 cohort and in only 2% of patients in the other two cohorts [43]. The phase 3 CheckMate 649 trial is currently recruiting metastatic gastric or EGJ cancer patients with or without PD-L1 expression to evaluate the efficacy of nivolumab plus versus oxaliplatin plus fluoropyrimidine as a first-line treatment (NCT02872116).

Avelumab

Avelumab is a monoclonal antibody against PD-L1. The phase 1b JAVELIN trial analyzed a cohort of patients with gastric and EGJ tumors. Patients received avelumab as first-line maintenance or a second-line treatment. A total of 151 patients received avelumab (10 mg/kg IV every 2 weeks). An unconfirmed response was observed in 9.0% of patients in the maintenance group and in 9.7% of patients who received the medication as a second-line treatment. The disease control rate was 57.3% and 29.0%, and the median PFS was 12 weeks and 6 weeks for the first-line maintenance and second-line treatment groups, respectively. Grade 3 or higher treatment-related AEs were observed in 9.7% of patients [49]. These results led to the development of phase 3 trials addressing avelumab as a first-line maintenance therapy (NCT02625610) and as a third-line treatment (NCT02625623) for metastatic gastric and EGJ cancers.

Novel Cytotoxic Drugs

Nab-paclitaxel (Nab-PTX) is a nanoparticle albumin-bound PTX which does not contain cremophor or ethanol as a formulation vehicle used for poorly water-soluble drugs. As a result, nab-PTX has a smaller risk of hypersensitivity reactions, and high doses can be administered over a short infusion time. ABSOLUTE is a Japanese phase 3 trial that showed non-inferiority of weekly nab-PTX to soluble-based PTX as second-line chemotherapy for advanced GC in terms of OS [50]. In contrast, non-inferiority of nab-PTX every 3 weeks to soluble-based PTX in OS was not confirmed with lower QoL scores.

DHP107 is a novel oral lipid formulation of paclitaxel. DREAM is a randomized phase 3 study for advanced GC after failure of first-line therapy to compare DHP107 and paclitaxel [51]. Non-inferiority regarding PFS was confirmed, although higher gastrointestinal toxicities are reported. A randomized phase 2 trial of S-1 plus leucovorin (TAS-118) versus S-1 plus leucovorin and oxaliplatin (SOL) versus S-1 plus cisplatin in advanced GC patients showed a higher response rate of SOL with a longer OS [52]. Currently, the phase 3 SOLAR study comparing TAS-118 plus oxaliplatin with S-1 plus cisplatin is ongoing in Asian countries.

TAS-102 is a novel oral nucleoside antitumor agent containing trifluridine and tipiracil hydrochloride, which prevents the degradation of trifluridine. Based on a phase 2 trial of TAS-102 for pretreated advanced GC with a disease control rate of 65.5% [53], the ongoing global phase 3 trial is investigating the efficacy and safety of TAS-102 in patients with advanced GC refractory to standard treatments.

Conclusions and Future Prospects

After years of stagnation in the medical treatment of GC, including numerous negative phase 3 trials investigating molecularly targeted drugs, eventually, some progress is emerging. This development is linked to our increasing knowledge of genetic alterations and molecular signatures in GC, as elaborated by The Cancer Genome Atlas consortium and other networks. A major limitation, however, is the biological heterogeneity, which is inherent to GC [54]. A major step forward is expected from immunotherapy. Anti-PD-1- and anti-PD-L1-directed agents, alone or in combination with anti-CTLA-4, show a promising activity. Appropriate molecular stratification of the population for targeted treatment remains challenging. Progress achieved with anti-angiogenic agents, namely, with the VEGFR-2-directed antibody ramucirumab in second-line treatment of advanced GC, was rather small. Now, first-line data are awaited, and the integration of ramucirumab in multimodal treatment concepts as well as combination with novel targeted agents like immune checkpoint inhibitors remains interesting. Other emerging therapeutic options comprise targeting of the tight junction protein Claudin-18.2, STAT-3-dependent gene expression as a cancer stemness-related pathway, and tumor stroma modification via inhibition of MMP-9.

Acknowledgments The authors thank Silvia Flora for manuscript editing assistance.

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8 Combined Modality Treatment for Locally Advanced Gastric Cancer: Current Evidences and New Perspectives

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Introduction

Gastric cancer still represents a major global health problem. In spite of a decrease in incidence and mortality rates during the last decades in Western countries, gastric cancer remains a common malignancy in regions such as Eastern Asia, Eastern Europe, and parts of South America making it the second leading cause of cancer death in the world [1]. Significant changes in the epidemiology of gastric cancer have also occurred. Compared to Eastern countries, the incidence of both proximal gastric and distal esophageal adenocarcinomas has risen in Western countries during the past two decades, with related diagnostic, prognostic, and treatment implications [2].

Apart from Japan, where mass screening programs have been developed for an early detection of this disease, most patients have advanced or unresectable tumor at diagnosis, and only less than half of them are likely candidates for curative surgery [3]. However, even when curative resection can be performed, the reported 5-year survival rate of 20–40% for stage II–III disease is still disappointing [4]. Locoregional recurrences are common after curative resection, remaining a substantial problem also in the modern surgical series; in addition, distant metastases occur, as a component of failure, in a significant part of recurred patients [5–7]. Extended D2 lymph node dissection has the advantage of accurate nodal staging, and it has demonstrated to improve cancer-specific survival but also to increase

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_8

surgical morbidity and mortality [8–10]. Because of the increased postoperative (postop) mortality, which has been related to splenic and pancreatic resections, several authors suggested D2 dissection without splenopancreasectomy for these patients [11], and a more recent Cochrane systematic review and meta-analysis support the non-inferiority of spleen preservation versus splenectomy, in terms of survival, in patients with proximal gastric cancer [12].

Despite years of randomized trials, the results of adjuvant chemotherapy (CT) still remain disappointing, and the more recent meta-analysis indications for survival benefit await confirmation by further trials using modern and potentially more active drug combinations [13, 14]. A major interest has emerged during the past few years in other adjunctive treatment modalities, resulting in different but beneficial approaches including postop chemoradiotherapy (CT-RT), perioperative (periop) CT, and preoperative (preop) CT-RT, which changed significantly the clinical practice and clinical research in gastric cancer.

This paper examines the current evidence from these various approaches and highlights the most recently reported clinical trials with focus on therapeutic options to further improve current treatment strategies for patients affected by this disease.

Combined Modality Treatment Options: First Generation of Randomized Clinical Trials

Adjuvant Chemoradiotherapy

Postop CT-RT has been the first strategy to provide a survival benefit in gastric cancer. The INT0116 trial demonstrated that postop CT-RT significantly improved relapse-free and overall survival, compared to surgery alone, in patients with locally advanced, stage Ib–IV M0, gastric or gastroesophageal (GEJ) cancer [15]. The CT-RT program consisted of one cycle of 5-fluorouracil (5-FU) and leucovorin for 5 days followed by CT-RT with 45 Gy and concurrent 5-FU/leucovorin on the first 4 and last 3 days of RT, followed by two further cycles of 5-FU/leucovorin. The survival at 3 years was 50% versus 40% ($p = 0.005$) in favor of the CT-RT group. The survival benefit was also maintained after a median follow-up of 10.3 years [16]. Despite these positive results, the study was criticized because of the low rate of D2 lymph node dissection, with 54% of patients having only a D1 lymphadenectomy, and because the survival rates in the Intergroup trial were not better than those observed in the negative European adjuvant trials. Moreover, grade 3–4 hematological and gastrointestinal toxicities occurred in 54% and 31% of patients, respectively, resulting in a limited compliance to treatment; only 64% of patients were able to complete postop RT-CT as planned.

As most patients had limited surgery and the impact on disease control and survival was mainly related to local control, it has been suggested that postop CT-RT compensated for inadequate surgery with the possible implications that such treatment could not be necessary in patients who have a more extensive D2 lymph node dissection and further investigations with this adjuvant approach were suggested.

Perioperative Chemotherapy

The MAGIC trial was the first trial to show an improvement in survival by periop CT in patients with gastric and GEJ cancers. The MAGIC trial consisted of three cycles of preop and three cycles of postop epirubicin, cisplatin, and infused 5-FU (ECF) that significantly increased the R0 resection rate compared to surgery alone (79% versus 69%, respectively) in operable gastric and GEJ cancers, stage II–IV M0. The CT arm showed a significant improvement in overall survival (36% vs 23% at 5 years, $p = 0.009$), downsizing (median tumor size 3 vs 5 cm, $p < 0.001$), and downstaging, both in the primary (T1–T2: 52% vs 37%, $p = 0.002$) and in nodal diseases (N1/N2: 84% vs 71%, $p = 0.01$). Notably, the few patients with EGJ cancer (11%) seemed to have more benefit from CT, suggesting a better tumor chemosensitivity. Rates of postop complications were similar in both groups of patients (46% and 45%, respectively) as well as the incidence of deaths within 30 days after

surgery (5.6% and 5.9%, respectively), which demonstrates the feasibility and safety of preop CT. However, only 55% of patients in the arm with periop CT began the postop treatment, mainly due to early disease progression, postop complications, or patient refusal. Overall, only 42% of patients completed the planned six-cycle program, suggesting a problematic patient compliance to treatment mainly related to poor general conditions after preop CT and surgery [17].

These results were confirmed in a second periop trial reported by the French FNCLCC/FFCD 9703 phase III study [18]. Compared with the other adjuvant and periop trials, in this study most patients had GEJ (64%) and lower esophageal (11%) tumors; only 24% of patients had gastric cancer. Patients were randomized to receive either two or three preop cycles of continuous infusion 5-FU and cisplatin (FP regimen) followed by surgery or surgery alone. Four cycles of postop FP were planned in case of response to preop CT or stable disease with positive lymph nodes. Periop CT resulted in significantly improved 5-year overall survival (38% versus 24%) and 5-year DFS (34% versus 21%). Similarly to the MAGIC trial, the R0 resection rate was significantly improved, and the subgroup of EGJ tumors showed the greatest benefit from preop CT, but only a part of patients were able to complete the postop component of treatment.

The feasibility and tolerance of preop compared to postop approach was addressed by the Swiss Group for Clinical Cancer Research (SAKK); the group reported the data of a randomized trial demonstrating that docetaxel-based preop CT is better tolerated than postop CT, thus confirming the indications on patient compliance and further supporting the preop approach [19].

Adjuvant Chemotherapy

While adjuvant CT trials from Japan and Korea showed a clear benefit of adjuvant therapy for stage II and III gastric cancer using oral S1 for 1 year or intravenous oxaliplatin and capecitabine (XELOX) after surgery [20, 21], European trials have been disappointing so far. Three randomized trials evaluating adjuvant CT compared with surgery alone showed 5-year survival rates ranging between 40% and 50%, with no significant difference between arms [22–24]. Similarly, the most recently reported ITACA-S trial, evaluating an intensive adjuvant regimen with sequential FOLFIRI followed by docetaxel plus cisplatin, failed to show any benefit in disease-free and overall survival versus monotherapy with 5-FU/leucovorin alone. Based on these results, 5-FU/leucovorin has been considered as the standard treatment for patients radically operated with D2 dissection [25]. A recent meta-analysis based on individual patient data suggested a small but defined benefit with adjuvant CT, which remained constant after testing for heterogeneity according to the geographic region where the study was conducted (Europe, Asia, North America) and the CT regimen administered (monotherapy or combination CT), supporting the evidence of its indication [13, 14].

Preoperative Chemoradiotherapy

For operable gastric cancer, an increasing interest has emerged more recently in the preop CT-RT approach as a three-step strategy with induction CT followed by preop CT-RT and subsequent surgery delayed up to 18–20 weeks. Recent advances in clinical staging, including endoscopic ultrasound and laparoscopy, are now available to identify patients who are potentially candidates for this innovative treatment strategy [25]. The CT-RT approach appears to be better tolerated in the preop setting, and it may increase the likelihood of an R0 resection, as reported with preop CT alone. In addition, as the primary tumor is still in place, the radiation planning can be more accurate compared with the difficulties experienced with postop CT-RT [15]. Some phase II studies have shown the feasibility of this approach including two cycles of induction CT with 5-FU and cisplatin followed by preop concurrent CT-RT with 5-FU and cisplatin or Taxol combined with 45Gy of radiation followed by radical gastrectomy [26, 27]. Pathological complete response (pCR) ranged from 20% to 30% and R0 resection rate from 70% to 78%; pCR and

Table 8.1 Combined modality treatment: first generation of RCTs

Trial	N° Pts	Treatment	R0	pCR	5ys OS
INT0116 Stomach, 80% GEJ, 20%	559	Postop FU/LV + RT-FU/LV vs surgery alone	100%	–	35% vs 27%
MAGIC-B Stomach, 74% GEJ, 11% ES, 15%	503	Periop ECX 3 + 3 vs surgery alone	68% vs 66%	None	36% vs 23%
FFCD/ACCO Stomach, 25% GEJ, 64% ES, 11%	224	Periop CF 3 + 3 vs surgery alone	84% vs 74%	None	38% vs 24%
RTOG 9904 Stomach,100%	43	Preop FU/LV-DDP x2 + RT-FU	77%	26%	–

R0 resection were found to be independent prognostic factors for survival in a pooled analysis of these studies, and an excellent 5-year survival rate was reported. Grade 4 toxicity occurred in 21% of patients [28]. The major concern with this approach is the risk of tumor progression during neoadjuvant treatment, with unresectable or metastatic disease at the postponed planned surgery. Available data reported an incidence of 16–25% of disease progression at surgery in patients treated with neoadjuvant CT or CT-RT [28]. Whether patients who recur during preoperative treatment indicate a subset with more aggressive and rapidly progressive disease, in whom no benefit from surgery can be expected, or a disease progression in nonresponsive patients, remains a matter of debate. The identification of this high-risk group of patients could be better addressed by the biological characterization of the disease. Advances in molecular biology are in progress and could result in a more accurate determination of prognosis and individualized therapeutic strategies. The first generation of randomized clinical trials on combined modality treatment is reported in Table 8.1.

Beyond INT0116 and Magic: Highlights of the Second Generation of Randomized Trials and Novel Clinical Approaches

The INT0116 and MAGIC trials changed significantly the clinical practice and clinical research in gastric cancer in the last decade. To answer the several questions that emerged from these landmark trials, a new generation of adjuvant/neoadjuvant gastric RCTs and novel neoadjuvant approaches were planned and have been recently reported as follows.

Is Postoperative Chemoradiotherapy Beneficial Also After D2 Lymphadenectomy?

A first attempt to answer the question of whether postop CT-RT is beneficial after D2 lymphadenectomy was made by the Korean Adjuvant Chemoradiotherapy in Stomach Tumors (ARTIST) phase III trial, which compared adjuvant CT-RT with two cycles of capecitabine-cisplatin before and after capecitabine-based CT-RT versus six cycles of adjuvant capecitabine-cisplatin alone [29]. The study was negative, even if a trend toward an improved 3-year disease-free survival (78.2% vs 74.2%, $p = 0.09$) was noted in the CT-RT arm. In addition, in a subset analysis of patients with lymph node-positive disease, the 3-year DFS was significantly improved with CT-RT (77.5% vs 72.3%, $p = 0.04$). These results were confirmed at a longer follow-up of 7 years. The ARTIST trial differed from Intergroup trial in two important ways: firstly, all patients had D2 dissection with a median number of 40 examined lymph nodes, and, secondly, the control arm had adjuvant CT; thus, comparison of the two studies is somewhat difficult. One can speculate that postop CT-RT was beneficial also after D2 dissection, but this benefit was neutralized by postop

CT. It is also possible that postop CT-RT was better than postop CT in node-positive patients, but this is based only on a subset analysis with its relative limitations.

The ongoing ARTIST II 3-arm phase III trial is evaluating the efficacy of postop CT-RT (S1-oxaliplatin followed by radiation 45Gy and capecitabine) versus postop CT (S1 or S1-oxaliplatin) in patients with lymph node-positive gastric cancer receiving D2 lymph node dissection [30].

Does the Addition of a More Effective Chemotherapy Component Improve the Efficacy of Postoperative Chemoradiotherapy?

As the improvement in overall and disease-free survival for patients who underwent postop CT-RT in the INT0116 trial was mainly related to an increase of local control rather than to a decrease in the incidence of metastatic disease, the Cancer and Leukemia Group B (CALGB) promoted a new phase III trial testing a modern and more effective CT regimen with one cycle of epirubicin, cisplatin, and 5-FU (ECF) followed by radiation of 45Gy and concurrent continuous infusion 5-FU and by two more cycles of ECF versus the treatment arm of the INT0116 study (45 Gy and concurrent 5-FU/leucovorin). The results of this trial have been recently reported and demonstrated that the addition of epirubicin and cisplatin in postop CT-RT is not superior to standard 5-FU/leucovorin in terms of disease-free and overall survival (37% vs 39% and 44% vs 44%, respectively). Also, no significant differences were observed for either locoregional recurrences or distant metastasis rates between treatment arms and any patient subgroup, including the extended lymph node dissection group (55% of patients had >15 lymph nodes and 11% had <7 lymph node examined) [31].

Although surgical treatment was more standardized compared to the previous Intergroup study, these results are quite different from those reported in the abovementioned postop CT-RT ARTIST trial with the reported survival rates >70%. Again, comparison of these studies is difficult because of several implications in different patient populations, CT-RT and CT regimens, and some details in surgical approach.

Does the Addition of Targeted Agents or New Chemotherapy Combinations Improve the Efficacy of Perioperative Chemotherapy?

Following the MAGIC trial, there has been intensive clinical investigation to further improve the perioperative approach. The addition of bevacizumab to epirubicin, cisplatin, and capecitabine (ECX) in a phase III recently reported STO03 trial failed to demonstrate its superiority to periop ECX alone in patients with resectable gastric and EGJ adenocarcinoma [32]. Progression-free survival and overall survival were similar in the study arms, as were the rates of pathological response, with a pCR of 5% and 7% for ECX and ECX-bevacizumab, respectively.

More recently, results from the phase II part of the German FLOT4 phase III trial showed that preop docetaxel, oxaliplatin, and FU/leucovorin (FLOT) increased the pCR rate compared with ECF or ECX (16% vs 6%, $p = 0.015$) in resectable gastric (48%) or GEJ adenocarcinoma (52%). Interestingly, this favorable activity in terms of pCR was more evident in the intestinal-type histology (16%) compared with diffuse-type histology adenocarcinoma (3%, $p = 0.004$) [33]. This data of activity of docetaxel-based CT has been also reported in the phase I–II study with docetaxel, oxaliplatin, and capecitabine (DOC) currently ongoing in our Institute in advanced metastatic disease [34]. In the FLOT4 trial, postop morbidity was 40% with ECF-ECX and 25% with FLOT ($p = 0.02$), and postop mortality was 4% with ECF-ECX and 2% with FLOT (p = NS). The last update of the study confirmed also a significant impact of FLOT regimen for 5-year overall survival (39% vs 33%, $p = 0.001$) [35]. Importantly, also in this trial, only 50% of patients in the ECF-ECX group and 61% in the FLOT group started postop CT, thus confirming the problematic patient compliance for postoperative treatment after preop CT and surgery.

In summary, the use of periop CT for locally advanced gastric and EGJ adenocarcinoma is a well-established approach; periop CT has shown to improve survival in both the MAGIC and the FNCLCC-FFCD trials. Subsequent studies have confirmed these results. While the addition of bevacizumab to ECX of MAGIC failed to demonstrate superiority to ECX alone, docetaxel-based triplet regimen (FLOT) was superior to the standard anthracycline-based regimen ECF-ECX (FOLT4 trial). As result, the FLOT regimen expands the current available periop CT options for the treatment of resectable gastric and EGJ adenocarcinoma, and it could represent the preferred histology-driven option for patients with intestinal-type tumors. Patient compliance for postoperative treatment after preoperative chemotherapy and surgery still remains a problem.

Does the Addition of Postoperative Chemoradiotherapy Provide Additional Advantage to Perioperative Chemotherapy?

The results of phase III CRITICS trial, which evaluated three cycles of preop epirubicin, cisplatin, and capecitabine (ECX) followed by radical gastrectomy with D1+ lymphadenectomy and either three addictional cycles of postop ECX or CT-RT with 45Gy and capecitabine-cisplatin, have been recently reported [36]. This was the first trial that directly compared the two standard of care strategies for adjuvant treatment in resectable gastric cancer defined in the last decade: postop CT-RT, as investigated in the INT0116 trial, and periop CT, as investigated in MAGIC trial. The trial was designed with appropriate quality assurance criteria resulting in high standard of surgery as demonstrated by high D1+ resection rate of 86% and R0 resection rate of 81%. In addition, a detailed radiation therapy quality assurance was also included, consisting in a real-time pretreatment planning central review.

The trial did not show any superiority for postop CT-RT compared with postop CT alone in patients having preop CT and adequate surgery. There was no difference in overall or event-free survival or, surprisingly, in locoregional control. Tolerability was similar in the two treatment arms. Furthermore, it should be emphasized that also in this trial only half of the patients in both treatment groups were able to complete the postop component of treatment as planned. This difficulty in delivering postop treatment, in particular after preop CT-RT, confirms the poor postop patient compliance previously reported in both INT0116 and MAGIC trials and the necessity to better optimize the timing of combined modality treatment with surgery.

Does the Addition of Preoperative Chemoradiotherapy to Perioperative Chemotherapy and Postponed Surgery Provide Additional Advantage?

As benefits have been demonstrated in trials with periop CT, postop CT-RT, and preop CT-RT, questions about patient selection and the optimal sequence of treatments in a possible integrated approach still remain open.

The ongoing TOPGEAR trial (Trial of Preoperative therapy for Gastric and Esophagogastric Junction Adenocarcinoma), an international phase III study, is testing the addition of CT-RT to periop CT by administering it in the preop rather than in postop setting. The study design allows a comparison of the MAGIC regimen with the INT0116 regimen but with the specific intent to move CT-RT into the preop setting.

Patients in the periop-alone group receive three cycles of ECF CT, while patients in the CT-RT group receive two cycles of ECF followed by radiation with 45Gy and concurrent FU. Both groups of patients will receive three more cycles of ECF after surgery. This study is based on the potential advantages of preop therapy for tumor downstaging, with an increase R0 resection rate, and the better tolerability of preop compared to postop therapy. Indeed, the study represents also an evolving strategy of the several phase II studies of preop CT-RT in gastric cancer which have

demonstrated safety, tolerability, and high rates of pathological response.

The recently reported interim analysis on the first 120 enrolled patients demonstrated that 93% of ECF group and 98% of CT-RT group patients received all cycles of preop CT and radiation as planned, while only 65% and 53%, respectively, received all cycles of postop CT. The proportion of patients proceeding to surgery was encouraging: 90% of patients in ECF group and 85% in CT-RT group underwent operation. Grade 3 or higher postop complications occurred in 22% of patients in both groups.

These results demonstrated that preop CT-RT can be safely delivered to the most part of patients after two cycles of induction ECF CT, without a significant increase in treatment toxicity or surgical morbidity. However, once again, also this study showed a limited patient compliance for postop CT after preop CT or CT-RT. The study is ongoing on the primary endpoint of overall survival, and results of the efficacy on adding preop CT-RT to periop CT are pending [37].

In order to further optimize preop CT-RT, a phase II study with a more effective systemic component including three cycles of induction CT (as MAGIC strategy) with new and potentially more active regimen including epirubicin, oxaliplatin, and capecitabine (EOX) [38], followed by preop CT-RT with modern drug-radiation combinations (3D-CRT or IMRT 45Gy and concurrent capecitabine and weekly oxaliplatin) and by standardized surgical procedure, postponed at the end of the overall neoadjuvant program (after 20–22 weeks), was activated at our Institute (Neoadjuvant Epirubicin, Oxaliplatin, Capecitabine, and Radiation Therapy, NEOX-RT study). Examples of structures and lymph node station delineation and IMRT plan are reported in Figs. 8.1 and 8.2 (NEOX-RT study). This was a multicentric phase II study aiming to assess the feasibility of a complete CT (three cycles) and CT-RT program both given preoperatively in order to improve patient compliance to the combined modality treatment and to further improve resectability, pCR, disease control, and overall survival in locally advanced, stage T3–T4 N0 or N+, gastric cancer. This approach had also the potential advantage that a greater proportion of patients could receive all components of the multimodality treatment. An accurate staging including laparoscopic examination and patient careful clinical monitoring with endoscopic ultrasound and FDG-PET during treatment to identify patients with early asymptomatic metastatic disease at diagnosis or rapidly progressive disease during treatment was planned.

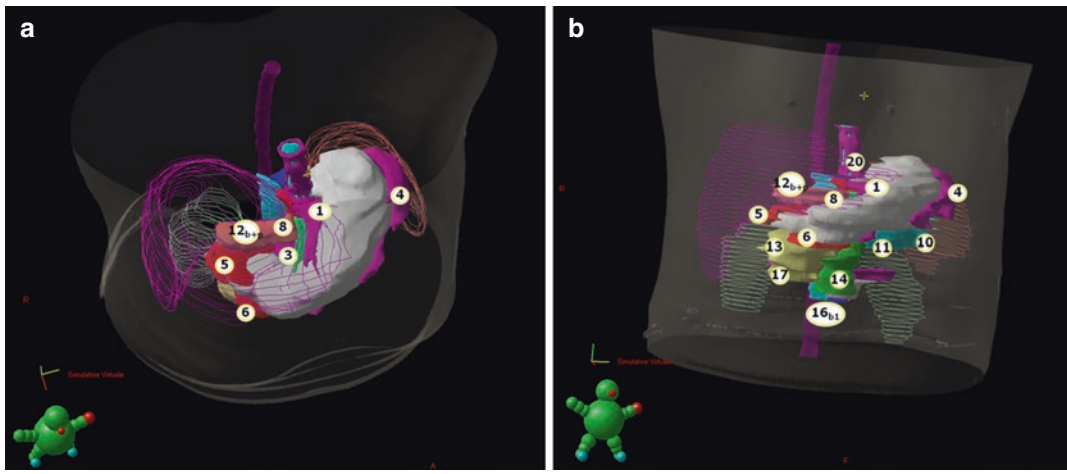

Fig. 8.1 Gastric cancer: structures and lymph node stations contouring in IMRT planning (NEOX-RT study). (**a**) Anterior 3D visualization. (**b**) Posterior 3D visualization (By courtesy of F.Cellini - GemelliArt, Roma)

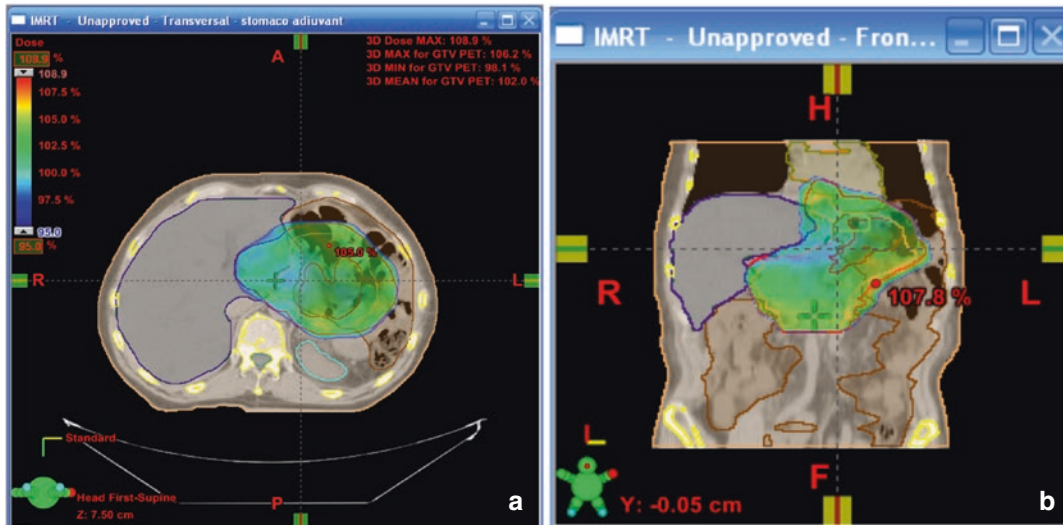

Fig. 8.2 Gastric cancer (antrum): IMRT plan for preop 45Gy/capecitabine-oxaliplatin (NEOX-RT study). (**a**) Target volume in transverse section. (**b**) Target volume in frontal section

In the reported interim analysis on the first 21 enrolled patients, tumor downstaging and nodal downstaging were 65% and 60%, respectively, with a pCR in 18% of patients. Compliance to treatment was good, and most patients completed the CT (87%) and CT-RT (86%) programs as planned. The most part of patients (90%) underwent R0 resection, and the median time to surgery was 23 weeks (planned 20–22 weeks). One patient had postop major complications. NEOX-RT appeared feasible and safe, and the pCR rate of 18% was remarkable [39]. The study has been recently concluded and final results will be reported.

Gastric and Gastroesophageal Junction Adenocarcinomas: Does Location and Histology Matter?

Because of different anatomic regions and lymph node compartments involved by the tumor, substantial differences between gastric and GEJ adenocarcinomas exist, with significant implications in the extension of surgical resection [40]. In addition, different epidemiological trends and histological characteristics between adenocarcinomas of these two anatomic regions have been also observed. GEJ cancer is a disease of smokers and drinkers. Gastric cancer is strongly associated with *Helicobacter pylori* infection, atrophic gastritis, and decreased acid production, while GEJ cancer is associated with a high acid production and Barrett's changes in the esophageal mucosa. Furthermore, tumors located in GEJ and cardia are predominantly of intestinal type (Lauren's classification) compared to distal stomach tumors, where diffuse types are more commonly located. While a decreased incidence of intestinal-type tumors in the distal stomach has been observed, the intestinal type, located in the proximal third, and the diffuse type at any location, have increased over time. Most importantly, tumor location and histology are associated to different clinical aggressiveness, treatment response, and prognosis. Proximal tumors, including GEJ, and diffuse histotype have usually a worse prognosis with a higher risk of lymph node and peritoneal spread [41]. However, GEJ tumors appear to be also the more responsive to preop CT(RT), and an unexpected high rate of pCR has been reported [33, 42, 43]. An important finding is that intestinal histotype tumors are more likely to achieve a pCR, compared to diffuse histotypes [33]. In addition, more rare histologic subgroups as signet-ring cell tumors,

existing in any gastric and EGJ location, demonstrated to be inherently resistant to the current chemo(radio)therapy regimen, and implications on treatment programs should be considered [44]. Interestingly, both intestinal and diffuse tumor types showed different targetable biomarker expression profiles such as Her2 in the intestinal and EGFR in the non-intestinal pathway, indicating new therapy options [45].

Optimal management of patients with GEJ adenocarcinoma is still a controversial issue [46]. No randomized clinical trials have specifically selected this subset of patients, and clinical data are derived from studies addressing both esophageal and gastric cancers, including either squamous cell carcinoma or adenocarcinoma [47]. Most part of these trials evaluated the impact of neoadjuvant CT-RT [48–50] or neoadjuvant CT [33, 51, 52] as compared to surgery alone. A recently updated meta-analysis confirmed a strong evidence for a survival benefit of neoadjuvant CT-RT or CT over surgery alone [42, 43].

The most solid evidence in favor of neoadjuvant CT-RT was provided by the CROSS trial comparing surgery alone to preop RT with 41.4Gy with concurrent carboplatin and paclitaxel followed by surgical resection [48]. Up to 75% of enrolled patients had adenocarcinoma, and 22% had a GEJ tumor. Overall, a pCR of 29% was reported, and this was more frequent in patients with squamous cell carcinoma (49% vs 23%, $p = 0.008$). Overall 5-year survival was 47% versus 34% in favor of preoperative CT-RT [53]. To further optimize this CT-RT program, a phase II study is currently ongoing at our Institute to evaluate feasibility and efficacy of intensified IMRT of 52.5Gy with the same concurrent CT. The recently reported initial results demonstrated the feasibility and safety and encouraging activity of the treatment [54].

Few studies compared neoadjuvant CT-RT versus CT prior to surgery. A first randomized study enrolled 119 patients with adenocarcinoma of the lower esophagus or cardia to receive two cycles of DDP-5-FU/LV or the same preop CT followed by 30 Gy of RT with concurrent cisplatin and etoposide. Aimed for 354 patients, the study was concluded early due to poor accrual. The pCR rate was significantly superior in the CT-RT group (15% vs 2%); although the rate of postop mortality was somewhat higher with CT-RT (10.2% versus 3.8%, $p = 0.26$), a 5-year survival showed a trend in favor of preop CT-RT (48% vs 40%, $p = 0.055$). Another trial compared two cycles of FU and cisplatin with 35Gy of radiation to neoadjuvant FU and cisplatin alone. Also this study concluded early for the low accrual. A superior pCR rate for CT-RT was reported also in this study (13% vs 0%), but without significant difference in OS [49]. However, when these data were combined with the pooled results of other neoadjuvant CT or CT-RT trials in recent meta-analysis, a trend in favor of neoadjuvant CT-RT was reported ($p = 0.07$) [43]. Importantly, no difference in morbidity was reported between the two treatment approaches. The second generation of selected randomized clinical trials on combined modality treatment is reported in Table 8.2.

In summary, based on the available evidence, both neoadjuvant CT-RT and CT provide significant survival benefits over surgery alone in patients with GEJ carcinoma. A clear advantage of neoadjuvant CT-RT over neoadjuvant CT, in the few studies including patients with GEJ adenocarcinoma, has not yet been established, and further trials comparing these two strategies should be promoted on this emerging and well-defined tumor site.

Conclusions

After years of negative studies, two successful treatment strategies with postop CT-RT (INT0116 trial) or periop CT (MAGIC trial) became available as standard of care for adjuvant therapy of resectable gastric cancer in Western countries [15, 17]. Because of some limitation of these studies, a second generation of clinical trials was planned in the last decade to answer several questions that emerged from these two landmark trials.

The efficacy of postop CT-RT also after D2 lymph node dissection has not been clearly demonstrated in the ARTIST trial; even if a significant advantage in the subset of patients with

Table 8.2 Combined modality treatment: second generation of RCTs

Trial	N° Pts	Treatment	R0	pCR	5ys OS
CALGB 80101 Stomach, 78% GEJ, 22%	546	Postop ECF + RT-FU + ECF vs postop FU/LV + RT-FU/LV	100%	–	44% vs 44%
ARTIST Stomach, 100%	458	D2 + postop X-P 2 + 2 + RT/Cape vs D2 + postop X-P x6	100%	–	77% vs 72% (3 years DFS)
STO03 Stomach, 36% GEJ, 50% ES, 14%	1063	ECX-bevacizumab vs ECX alone	61% vs 64%	7% vs 5%	48% vs 50% (3ys)
FLOT4 Stomach, 43% GEJ, 57%	265	Periop FLOT 4 + 4 vs periop ECF 3 + 3 or ECX	85% vs 74%	16% vs 6%	39% vs 33%
CRITICS Stomach, 83% GEJ, 17%	788	Periop ECX x3 vs periop ECX x3 + postop RT/X-P	80% vs 82%	6% vs 6%	42% vs 40%
TOPGEAR[a] Stomach, 73% GEJ, 27%	120	Periop ECF 2 + 3 + preop RT-FU vs periop ECF 3 + 3 alone	–	–	–
POET[b] GEJ, 100%	119	Preop FU/LV-DDP x14 weeks + preop RT/DDP-VP16 vs preop FU/LV-DDP x14 weeks	96% vs 84%	16% vs 2%	39% vs 24%
CROSS[c] GEJ, 25% ES, 75%	366	Preop RT/Carbo-PTX vs surgery alone	82% vs 59%	23% vs na	47% vs 33%

[a]Interim data
[b]Stopped for poor accrual
[c]*SCC* 23%; Adenoca 75%

positive lymph nodes has been reported, the trial was negative [29]. In addition, both the CALGB and the CRITICS trials, in which a more standardized D2 dissection was provided, failed to demonstrate an advantage of postop CT-RT with an added more effective systemic CT component [31] or when compared to post-op CT only in a periop approach [36]. However, the 3–5-year survival rates of these two trials were well comparable or better than the treatment arms of the original INT0116 and MAGIC trials, confirming the superiority of postop CT-RT and periop CT to surgery alone (Tables 8.1 and 8.2).

While the addition of target therapy (bevacizumab) to periop ECF(ECX) failed to demonstrate superiority to standard ECF(ECX) [32], the docetaxel-based triplet FLOT was superior to triplet epirubicin-based CT in the periop FLOT4 trial in terms of pCR, postop morbidity, and survival [33, 35]. These results confirm the initial data of the DOC phase I–II study currently ongoing at our Institute, and docetaxel-based CT expands the current available periop options in locally advanced, resectable gastric cancer [34].

The more recent growing interest in preop CT-RT is further supported by the interim results of the TOPGEAR trial which demonstrated the feasibility and safety of delivering preop CT-RT after two cycles of ECF CT with high compliance rates to treatment (98%) and the high proportion of patients proceeding to surgery (85%) [37].

However, there are still open questions regarding the duration of the preop CT component, and whether the postop CT component is necessary. As in other recent periop trials, also in TOPGEAR trial, only about half of the patients were able to receive CT after surgery.

Considering the better patient tolerance of preop treatment, future studies should focus on the possibility to move a more complete CT component in the preop period. On this issue, the

NEOX-RT phase II study was activated at our Institute which included a more effective neoadjuvant component with three cycles of EOX (with no postop CT) followed by CT-RT, and surgery postponed at week 20–22 could be a useful contribution for a new preop treatment approach in locally advanced gastric cancer. Interim analysis demonstrated the feasibility and safety of this prolonged treatment time before surgery, and the final results of the study will be reported [39].

Future studies also need to give more focus on GEJ and stomach cancer as separate diseases to provide more solid data in these established tumor types. Furthermore, defined histologic subtypes (i.e., intestinal, diffuse, or mixed histotypes) are associated to different aggressiveness, treatment response, and prognosis. Therefore, future clinical studies should take into account these emerging evidences. New current insights into histological patterns [55–57] and molecular characterization of individual tumor subtypes [58–60] could address new and more individualized generation of combined modality treatment programs.

Acknowledgments The authors thank S. Flora and D. Michilin for manuscript editing assistance.

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Surgical Strategies in Gastric Cancer

9

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Introduction

Surgical resection is the principal therapy for gastric cancer, as it offers the only potential for cure. Moreover, in the era of multimodality treatment, surgery plays a central role in the management of gastric cancer including staging evaluation, curative treatment, and when necessary palliation.

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Staging

In patients with gastric cancer, accurate staging is of main importance in order to plan optimal treatment strategies. Peritoneal carcinomatosis is one of the most frequently observed incurable factors in patients with gastric cancer. The prognosis of patients with peritoneal dissemination is dismal, and chemotherapy is indicated as first option. Peritoneal metastases are documented in up to one third of patients with previous negative staging abdominal CT scan. Staging laparoscopy allows for better definition of peritoneal spreading status by means of direct visualization, biopsy, and peritoneal washing cytology. Current indications for staging laparoscopy vary according to different societies' recommendations ranging from patients with cT3–cT4 tumors without evidence of lymph node or distant metastasis on CT scan to all patients with resectable gastric cancer [1–4]. Since chemotherapy and chemoradiotherapy regimens and indications depend on metastatic status, our indications for staging laparoscopy comprise all cases at risk of peritoneal spread including patients with doubtful cT2 tumors on endoscopic ultrasound and patients with cT3–cT4 N−/+ without evidence of peritoneal and distant metastasis on abdominal CT scan. In clear cT1–cT2 tumors on endoscopic ultrasound, we consider laparoscopy as initial procedure at the time of resective surgery in order to rule out peritoneal involvement, in which cases

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_9

gastric resection is postponed to neoadjuvant treatment and restaging.

During staging laparoscopy peritoneal washing for cytology evaluation is also recommended. Since patients with positive peritoneal cytology are at high risk of peritoneal failure and oncologic outcome is poor when gastrectomy is performed as first step, neoadjuvant treatment and subsequent restaging are recommended in these patients [5].

Moreover, in patients with positive peritoneal cytology without the presence of peritoneal carcinomatosis, hyperthermic intraperitoneal chemotherapy (HIPEC) appears to increase overall survival rates. However, the efficacy of HIPEC as part of multimodality treatment approach in patients with gastric cancer should be tested in randomized clinical trials. HIPEC-related systemic drug toxicity should also be considered [6, 7].

Curative Treatment

The ultimate goal of gastric cancer treatment is radical surgical resection which offers the best chances for long-term survival. The extent of stomach resection and lymph node dissection are the two main issues to be considered when planning radical surgery for gastric cancer. Since macroscopically (R2) and microscopically (R1) positive resection margins are negative prognostic factors, the type of surgical procedure (total gastrectomy vs. partial gastrectomy) is determined by the site of the primary tumor in relation to the extent of free gross resection margins required to minimize the risk of positive microscopic resection margins. Depending on different guidelines, gross margin of resection is considered to be adequate which varies from 4 to 5 cm. Moreover, due to its more aggressive behavior, a wider free gross resection margin up to 8 cm has been suggested in cases with diffuse histologic tumor type. However, this implies a total gastrectomy for most of the patients with diffuse-type tumors [3, 8]. Intraoperative frozen section for resection margin evaluation is recommended; however conversion into a more extensive procedure (subtotal to total gastrectomy or from total gastrectomy to gastroesophagectomy) should be balanced with the stage of disease, the potential increased morbidity to the patient, and the potential oncologic benefit [9].

The extent and number of lymph node dissection during gastric resection procedures has both staging and curative implications. The *AJCC Cancer Staging Manual*, 8th edition, recommends that at least 16 regional nodes be assessed pathologically but that removal/evaluation of 30 or more nodes is desirable [10]. Adequate number of examined lymph nodes is important since it minimizes stage migration allowing for more precise staging and consequently better prognostic assessment [11]. The recommended extent of lymph node dissection is D2 lymphadenectomy (Fig. 9.1) which is an extended lymph node dissection, entailing removal of nodes along the hepatic, left gastric, celiac, and splenic arteries, as well as those in the splenic hilum (stations 1–12a). Moderate evidences based on a recent Cochrane meta-analysis including five randomized trials of D1 versus D2 dissection, comprising the 15-year follow-up data of the Dutch trial indicate that there is a significant difference favoring D2 lymphadenectomy in disease-specific survival, but not in overall survival or disease-free survival. Moreover, data suggested no significant difference in OS between more extended lymph node dissection (D3 lymphadenectomy) and D2 lymphadenectomy [12, 13].

Moreover, in spite of most studies reporting higher postoperative morbidity and mortality rate with D2 lymphadenectomy (especially if splenectomy is performed), it has been demonstrated that D2 dissection can be done with low operative mortality, similar to that of a D1 dissection [14].

Laparoscopic total and distal gastrectomy for gastric cancer has been demonstrated to be technically feasible and to result in lower complication rate compared to open surgery. Moreover, D2 lymphadenectomy can be equally obtained using laparoscopy. However, while in stage I gastric cancer oncologic outcome has been reported to be comparable between open and laparoscopic surgery, long-term oncologic data from prospective randomized trials in more advanced stages are not yet available [15–17].

At initial staging, unresectability criteria for gastric cancer are the presence of distant metastases, invasion of a major vascular structure, such

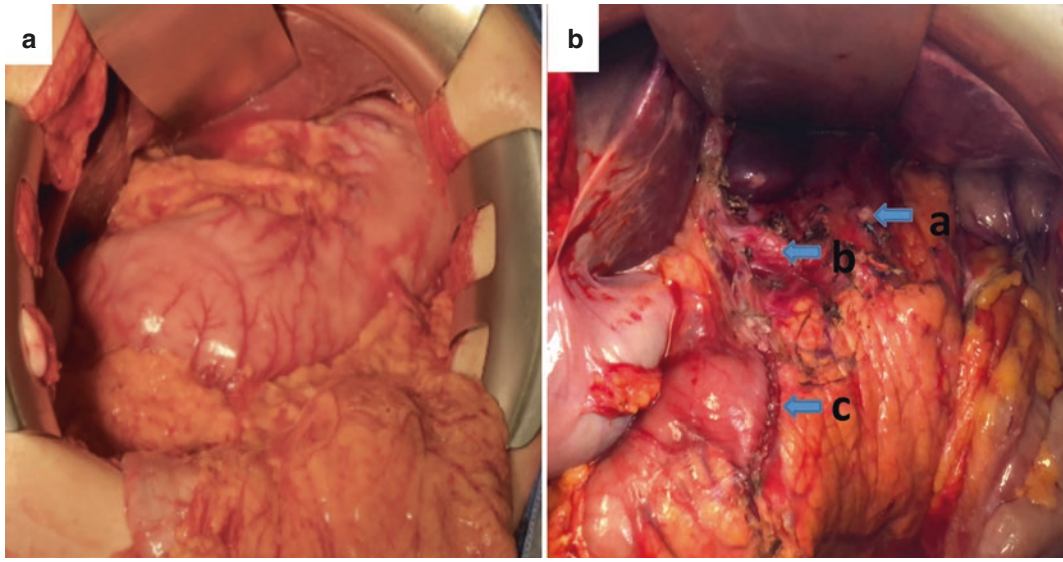

Fig. 9.1 (**a**, **b**) Adenocarcinoma of the greater curvature: (**a**) operative findings at laparotomy and (**b**) after total gastrectomy and lymphadenectomy (**a**) sectioned left gastric artery, (**b**) common hepatic artery, (**c**) duodenal stump

as the aorta, hepatic artery, or celiac axis. Conversion preoperative multimodality treatment including chemoradiotherapy with or without induction chemotherapy has been proposed for patients with locally advanced, initially unresectable, but nonmetastatic gastric cancer. Using this approach, potentially curative resectability rate as high as 70% has been reported with pathological complete response rates ranging from 5% to 30%. In these patients, one noncurative factor at initial diagnosis, complete pathological response, and R0 resection are all associated with favorable oncologic outcome. Therefore, initial staging, clinical evaluation after treatment, and extent of surgery aiming at curative resection are crucial for maximizing conversion treatment efficacy. In order to be adopted as a standard, this approach needs to be prospectively validated in clinical trials [18–21].

Gastric cancer presenting with a linitis plastica pattern (about 5% of all gastric cancer cases) is usually associated with diffuse histologic tumor type, peritoneal and distant metastasis, and a poor oncologic outcome. However, in the subset of nonmetastatic patients with linitis plastica amenable of radical resection, long-term survival rate similar to that of patients without linitis plastica has been reported. Therefore, optimal management of patients with linitis plastica should include accurate staging, neoadjuvant treatment, accurate restaging including laparoscopy, and intraoperative frozen section-guided radical surgery for achieving negative resection margins along with adequate lymphadenectomy [22, 23].

Liver Metastasis

Gastric cancer patients develop synchronous or metachronous liver metastasis in 5–14% of cases [24–28]. This disease has a dismal prognosis, and the median survival of patients with metastatic gastric cancer is approximately 6 months even with palliative chemotherapy [27, 29]. The role of surgical approach and hepatic metastases resection is controversial with some studies showing a doubtful effect on survival, while others reporting an improved outcome [30–39]. Rarely hepatic lesions are isolated, while in approximately 40% of cases, they are associated with peritoneal or extensive lymph node disease. The average survival following liver resection for metastasis from gastric cancer varies widely between 15% and 77% at 1 year and between 0% and 38% at 5 years with a median survival time of 5–31 months [25, 26, 28, 30, 35, 36, 38, 39]. Survival is significantly higher in case of single metastasis compared to multiple lesions and in metachronous compared

to synchronous metastasis [25, 26, 39]. Therefore, in highly selected cases of single metachronous gastric cancer, liver metastasis, metastasectomy, or ablative procedures may be considered.

Palliative Treatment

Most patients with gastric cancer at the time of diagnosis are not amenable of surgical treatment with curative intent, and many of them will require a palliative treatment during the course of the disease. The role of palliative surgery resection is very questionable. Systemic chemotherapy is the treatment of choice for patients with not resectable gastric cancer, and it has been proven that it improves both quality of life and survival providing a median survival of approximately 10 months [40, 41]. Unfortunately, chemotherapy is not equally effective in palliation of symptoms and complications such as vomiting, pain, occlusion, bleeding, and perforation. Patients with unresectable locally advanced gastric cancer or metastatic gastric cancer require a multidisciplinary approach that includes radiotherapy and endoscopic and surgical techniques such as palliative resection and gastrojejunostomy. External beam radiotherapy (RT) has a clearly defined role in controlling pain, bleeding, and occlusion in patients affected by unresectable localized gastric neoplasia [42–44]. There are no controlled studies comparing the RT with endoscopy or surgery, but in case of gastric outlet obstruction, the response to radiotherapy is not immediate as in the case of endoscopic stenting or palliative surgery. Furthermore, the required dose for the treatment of occlusive status is greater than 40 Gy and is normally associated with more side effects [44]. Endoscopic stenting is an excellent alternative for the treatment of obstruction. A systematic review has shown that endoscopic stent has the same effectiveness of palliative gastrojejunostomy but is associated with reduced hospitalization and a faster relief of obstructive symptoms [45]. On the other hand, patients undergoing endoscopic treatment require more often reintervention than patients undergoing palliative bypass surgery. Finally, endoscopy with laser photocoagulation or with application of a hemostatic nanopowder (Hemospray) remains the established treatment of bleeding control in the first instance [46, 47].

In patients with locally advanced or metastatic gastric cancer, the role of palliative resection remains controversial. A French retrospective survey has identified four predictive factors of survival in these patients: the ASA score (I or II), incomplete resection without metastasis or carcinomatosis, single-site solid organ metastasis without peritoneal carcinomatosis, and localized carcinomatosis without signet ring cells without histology. Palliative surgery should not be supported in case of high-risk patients (ASA III or IV) or in case of diffuse carcinomatosis or solid organ metastasis associated with carcinomatosis or finally limited carcinomatosis but signet ring cell histology [48]. In the phase III REGATTA trial, 176 patients with locally advanced gastric cancer associated with hepatic lesion or peritoneal lesion or para-aortic lymphadenopathy were randomized to chemotherapy alone or gastrectomy followed by chemotherapy. The overall survival was not significantly improved by surgery, and the study was closed after an interim analysis. The 2-year survival with chemotherapy alone was 32% while with surgery followed by chemotherapy 25%. Moreover chemotherapy following gastrectomy was associated with a greater incidence of adverse events such as leukopenia and hyponatremia [49]. Palliative laparoscopic or open gastrojejunostomy is indicated in case of gastric cancer associated with obstruction; however it is indicated only in those cases where endoscopy or radiotherapy is not possible [50]. In conclusion, the impact of palliative surgery on symptomatic relief and survival should be balanced with morbidity and mortality. Patient and tumor characteristics should be considered in order to select the optimal candidate. However, both palliative gastrectomy and gastrojejunostomy are not recommended in most of the cases.

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Part IV

Evolving Treatment Landscape in Gastric Cancer

10 From Molecular Classification to Targeted Therapy for Gastric Cancer in the Precision Medicine Era

Lara Alessandrini, Melissa Manchi, Fabrizio Italia, Tiziana Perin, and Vincenzo Canzonieri

Introduction

Gastric cancer (GC) is a common malignant neoplasm worldwide and one of the main causes of cancer-related deaths [1]. Despite some advances in therapies for GC, the long-term survival of patients with advanced disease remains poor. Historically, different types of classification have been used to stratify patients with GC for shaping the prognosis and treatment planning: anatomical classification (Borrmann classification and Siewert and Stein classification) [2, 3], histological classification (WHO classification and Lauren's classification) [4] and extent of disease (early gastric cancer vs advanced cancer) [5, 6]. More recently, the clinical impact of conceiving GC heterogeneity at diagnosis rather than a single disease has become evident. Therefore, based on new knowledge of molecular pathways associated with different aspects of GC, new pathogenetic classifications for GC have been and continue to be proposed. An improvement in the prognostic classification for GC is essential to develop a proper therapy for a selected patient population. The aim of this chapter is to discuss the state of the art on combining histological and molecular classifications of GC to provide an overview of the emerging therapeutic possibilities connected to the latest discoveries regarding GC.

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Development of Histological and Molecular Classifications of GC

Tan et al. [7], based on the genomic signature found in GC cell lines and patient tissues, classified GC into two major subtypes that overlapped with the histological Lauren classification. The G-INT subtype is related with intestinal histology, and the G-DIF is related to diffuse histology.

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_10

G-INT is characterized by the deregulation of genes associated with carbohydrate metabolism. Accordingly, the FUT2 gene encoding the galactoside 2-alpha-L-fucosyltransferase 2 enzyme affects the Lewis blood group involved in *H. pylori* infection, the LGALS4 gene encoding galectin 4 is implicated in the interaction between cell-cell and cell-matrix and the peptide transporter cadherin-17 is encoded by the CDH17 gene.

G-DIF exhibits deregulation of genes related to high cell proliferation and a high energy requirement. The AURKB gene encoding Aurora B kinase, which functions in the attachment of the mitotic spindle to the centromere, and the ELOVL5 gene, encoding elongation of very long-chain fatty acid protein 5, are examples of alterations in G-DIF. Tumours of the G-DIF type show a poor prognosis and a reduced response to chemotherapy compared with those of the G-INT type. Moreover, in vitro cell lines from the G-INT tumour type are more sensitive to 5-FU and oxaliplatin, while G-DIF tumours are more sensitive to cisplatin [7, 8].

Subsequently, in 2013, Singapore researchers categorized GC into three main types based on their genomic profiles [9]:

1. A profile characterized by a high proliferating number of cells with high genomic instability and *TP53* gene mutation
2. A metabolic profile associated with higher anaerobic glycolysis instead of mitochondrial oxidative phosphorylation to generate the energy (a phenomenon known as the "Warburg effect") and resulting in tumour cells more sensitive to 5-FU therapy
3. A mesenchymal stem cell profile with a high capacity for self-renewal, immunomodulation and tissue regeneration showing a sensitivity to PIK3CA-mTOR pathway inhibitors

Thereafter, The Cancer Genome Atlas (TCGA) research group, comprising mainly of Western Europe and US members, introduced the new technologies of large-scale genome sequencing analyses (i.e. copy number variation (CNV), exome sequencing, DNA methylation profile, mRNA and micro-RNA sequencing) to further classify GC into four main groups [10] (Fig. 10.1):

I. Epstein-Barr virus (EBV)-positive cancers (9% of all GC) characterized by DNA hypermethylation, a high frequency of PIK3CA mutations and PDL1/PDL2 overexpression

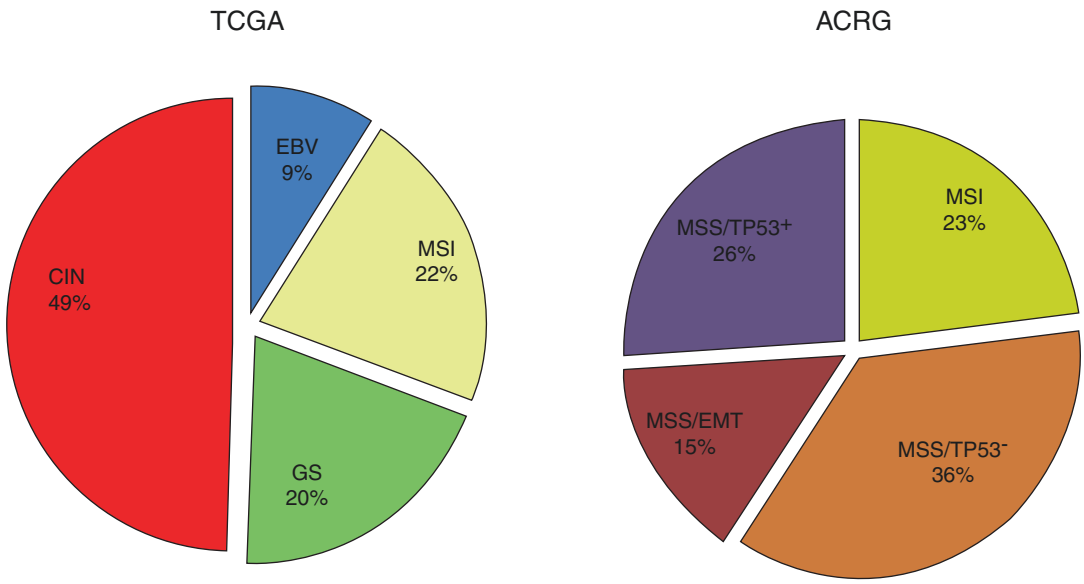

Fig. 10.1 Comparison of the different frequency rates of the molecular subtypes in TCGA and ACGR classifications

II. Microsatellite unstable tumours (MSI, 22%) showing a very high number of mutations and DNA methylation sites
III. Chromosome unstable tumours (CIN, 50%) mainly encoding alterations in tyrosine kinase receptors
IV. Genome stable tumours (GS, 20%)

In 2015, using similar approaches, the Asian Cancer Research Group (ACRG) also proposed a molecular classification for GC comprising four groups [11] (Fig. 10.1). They also proposed an MSI group (22.7%) but divided the remaining tumours based on the evidence of epithelial-mesenchymal transition (EMT) and p53 mutations (15.3% MSS/EMT microsatellite stable and EMT-associated tumours, 26.3% MSS/TP53+ active/intact and 35.7% MSS/TP53- inactive/altered tumours). Using these analyses, the MSI subtype had the best prognosis, while the MSS/EMT subtype had the worst. The former occurred predominantly at an early stage in the distal part of the stomach, showing mainly an intestinal histology (according to Lauren classification); the latter occurred at an advanced stage, at a younger age and with diffuse histology (>80%) and seeding in the peritonea with malignant ascites (64.1%) and had a frequency of 15–24% in the other subtypes. By contrast, liver metastasis was dominant in the MSI and MSS/TP53- types (approximately 20%). EBV infection was more frequent in the MSS/TP53 active group.

Similarities and Differences Between TCGA and ACRG Classification

ACRG validated their molecular sub-classification system using TCGA [10] and the Gastric Cancer Project '08 Singapore datasets [11]. The ACRG categories showed a significant overlap with TCGA subtypes: (i) regarding the tumours with an MSI profile, both classifications normally showed deregulation of the KRAS, NRAS and/or MLH1 gene; (ii) the enrichment of tumours with diffuse histology occurred more frequently in both the GS (TCGA) and MSS/EMT (ACRG) subtypes; (iii) both the EBV+ (TCGA) and MSS/P53+ (ACRG) subtypes showed PIK3CA and ARIDIA and rarely P53 mutations; and (iv) P53 mutations were often found in both CIN (TCGA) and MSS/P53- (ACRG) subtypes. However, ACRG did not classify tumours according to EBV status; CDH1 and RHOA mutations were rarely found in ACRG classification.

When the overall survival parameter was compared using TCGA and ACRG subtyping, only ACRG showed a significant association; TCGA classification only confirmed the association between better survival and the MSI subtype [12]. Nonetheless, presently, both TCGA and ACRG classifications raised sufficient potential to be used in clinical practice.

Limits of TCGA and ACRG Classifications

These novel classifications create a new paradigm in the definition of cancer biology and allow the identification of relevant genomic subsets using different techniques such as genomic screening, functional studies and molecular or epigenetic characterization. However, some limitations should also be openly recognized. First, these classifications are based on a highly complex methodology, and currently, they should not be replicated in standard laboratories lacking in the most recent technologies. Attempts towards simplification are ongoing, although the results may not fully capture the underpinning complexity of the disease. Second, these classifications lack prospective validation on a large scale, including patients of different ethnicities and age. Third, the two proposed classifications show more differences than similarities; in particular, they are different in terms of demographics, baseline molecular mechanisms, driver genes and association with prognosis. Moreover, there are notable dissimilarities in the distribution of Lauren's diffuse subtype among the different subgroups. Because different molecular subgroups may be identified across several independent gene expression profile studies, a collaborative international effort is warranted to aggregate a consensus classification. Fourth, the follow-up of included patients is limited, a factor that may decrease their prognostic power, and

subgroups were evaluated on resected specimens, with a different prevalence of subgroups among localized, locally advanced and advanced settings. Fifth, both classifications require epithelial cells, but none considers active, non-malignant stromal cells. Not only gene expression profiles derived from stromal tissues may influence the assignment to a specific molecular category, thus creating interpretative challenges [13], but also novel stromal-based distinctive signatures have been proposed and are related to the predominant cancer phenotype [14].

Integrated Molecular Signatures to Discriminate Intestinal and Diffuse Histological GC Subtypes

Previous findings have indicated that diffuse GC and intestinal GC might be two distinct diseases with a different molecular basis, aetiology, epidemiology and response to therapies. Molecular profiling in a recent study based on 300 GC cases identified 40 genes specifically expressed in diffuse or intestinal GC [15]; among them, three genes were independently associated with the patient's prognosis (for diffuse GC, EFEMP1 encoding an extracellular matrix glycoprotein and FRZB encoding a secreted protein involved in the regulation of bone development, which could also influence Wnt/β-catenin signalling; for intestinal GC, KRT23, a member of the keratins, which is responsible for the structural integrity of epithelial cells).

Several gene expression profiles of GC were also analysed, but the prediction accuracy of these methods resulted in lower gene signatures [16]. In the last year, a 9-gene signature including two negative impact factors (NR1I2 and LGALSL) and seven positive ones (C1ORF198, CST2, LAMP5, FOXS1, CES1P1, MMP7 and COL8A1) was proposed as a potential useful classifier to predict the outcome of GC, and the model clustered patients well into high- and low-risk groups with significant differences in both survival time and reoccurrence [17].

Although molecular characterization studies have attempted to identify prognostic gene signatures in GC, they are inadequate and fail to accurately guide patient therapy. Identifying tumour markers or constructing featured gene models are still the focus of many studies.

TCGA Classification of GC and Related Signalling Pathways Targetable for Precision Therapy

The four molecular subtypes of GC identified by TCGA classification are detailed below (see also Fig. 10.2), along with potential targetable pathways for precision therapy (Fig. 10.3).

EBV-Related GC

The EBV subtype (9% tumours in TCGA) is characterized by a high EBV burden [10]. EBV-positive tumours are more frequently located in the gastric fundus or body, and 81% of cases occur in men. In addition, EBV-positive GC is more prevalent in younger patients than in older subjects (Fig. 10.2). The histology of EBV-related GC is poorly to moderately differentiated adenocarcinoma, often accompanied by dense lymphocytic infiltration [18–21]. Relevant targetable pathways identified in this subtype are related to the elevated expression of programmed death ligands 1 and 2 (PD-L1 and PD-L2), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (PIK3CA) mutation and Janus kinase 2 (JAK2) amplification.

PD-L1 is a ligand of programmed cell death protein 1 (PD-1), which is expressed on T cells. PD-L1, which is expressed on tumour cells or stromal immune cells, inhibits the activation of cytotoxic T cells through an interaction with PD-1 and helps cancer cells to evade antitumour immunity [22–24]. Because the expression of PD-L1 is observed in many malignant tumours and is associated with a poor prognosis, PD-L1 has been studied extensively as a therapeutic target. Several studies have demonstrated that PD-L1, expressed on cancer cells or tumour-

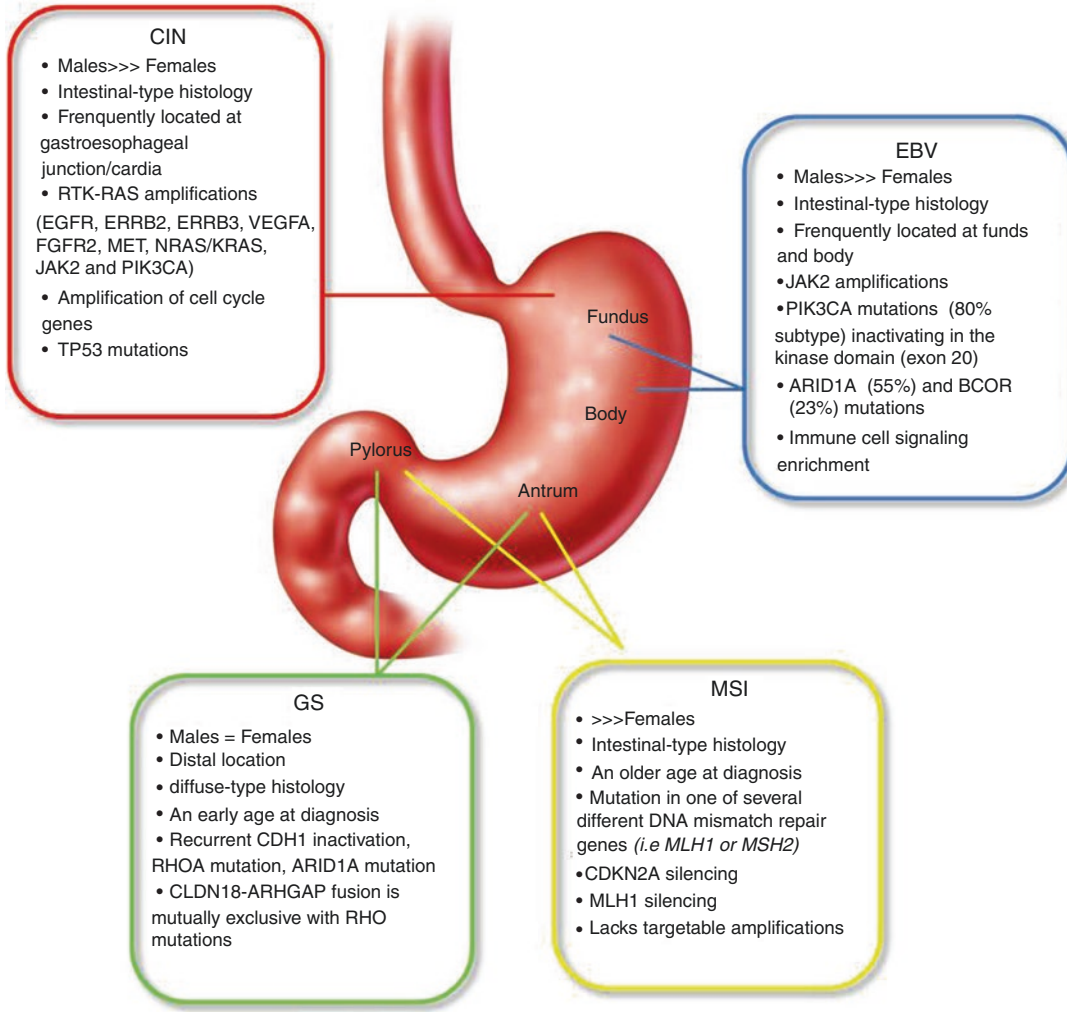

Fig. 10.2 The most relevant clinical-pathological and molecular features of TCGA subtypes

infiltrating immune cells, is a prognostic factor in GC, but the significance of PD-L1 in EBV-related GC has not yet been clarified [25–29]. In a recent study [30], both the expression of PD-L1 in cancer cells and PD-L1+ immune cell infiltration in EBV-related GC were significantly correlated with diffuse histology according to Lauren's classification and tumour invasion (pT1b or more). Therefore, this specific subtype of GC is potentially a good candidate for immunotherapy targeting of the PD-L1/PD-1 axis. Pembrolizumab, a highly selective immunoglobulin G4k humanized monoclonal antibody targeting the PD-1 receptor, demonstrated activity in the phase Ib KEYNOTE-012 trial in a cohort of heavily pretreated Asian and non-Asian patients with GC, with an acceptable toxicity profile [31]. On central review, the overall response rate was 22% [95% confidence interval (CI): 10–39%] with a median duration of response of 24 weeks (range: 8–33 weeks). Because most responses to chemotherapy in GC are short-lived, this is of significant interest. A correlation between PD-L1 expression (defined as PD-L1 $\geq$1% on archival tissue) and a response was subsequently demonstrated [32]. A double-checkpoint inhibition strategy targeting both the PD-1/PD-L1 and CTLA-4/B7 interactions, which has already

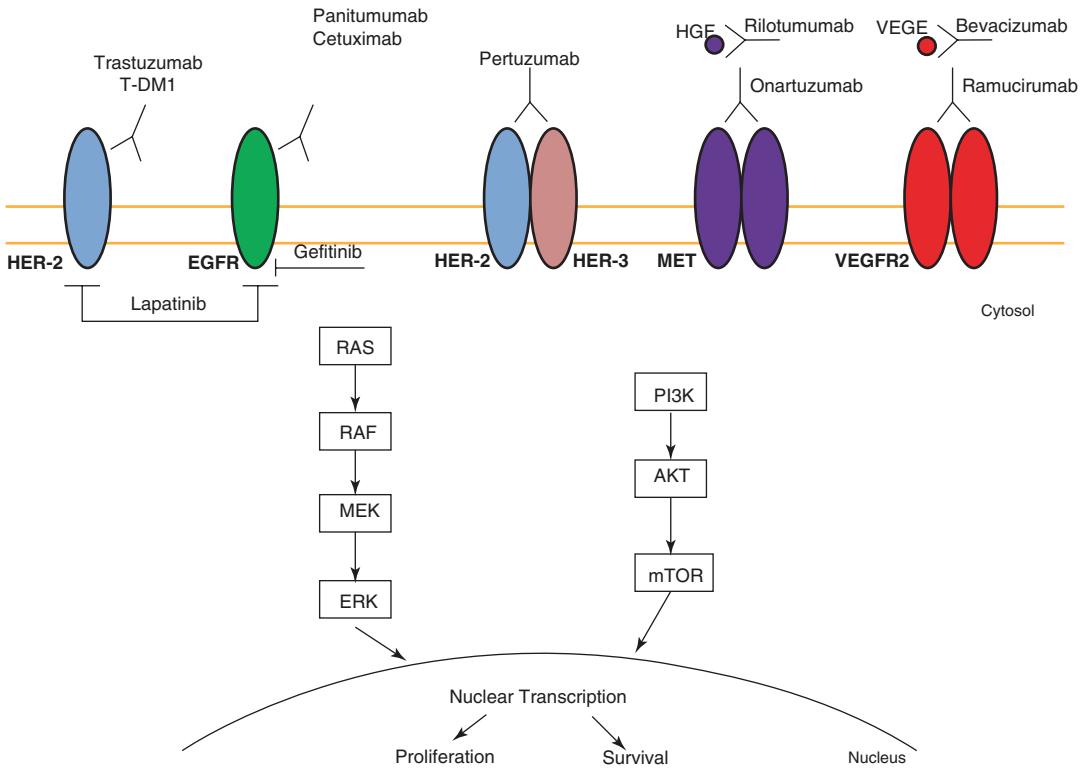

Fig. 10.3 Potential targetable pathways for precision therapy in GC addressed in the review

demonstrated efficacy in patients with melanoma, is under evaluation in several GC trials such as those using nivolumab + ipilimumab [ClinicalTrials.gov identifier: NCI01928394] and MEDI4734 and tremelimumab [ClinicalTrials.gov identifier: NCI02340975] [33].

The PI3K family of intracellular kinases mediates the regulation of cell survival, proliferation, differentiation, migration and metabolism [34]. The PI3K/AKT/mTOR pathway is frequently activated in GC, with the overexpression of *PI3KCA* described in 35–80% of GC cases [26–28] and phosphorylation of AKT described in 40–82% of GC cases [35, 37–39]. Expression of PI3KCA and phosphorylated AKT has also been associated with lymph node metastasis [35, 36, 38]. Furthermore, alterations in *PIK3CA* have been detected in 80% and 42% of the EBV and MSI molecular subtypes of GC, respectively [10]. The molecular mechanisms involved in the sensitivity to PI3K inhibitors are yet to be clarified to translate preclinical activity into a clinical benefit; to date, the development of PI3K inhibitors in advanced GC is still in the preclinical stage [40]. In gastric cancer, the PIK3CA mutation is an important biomarker for predicting the treatment response of everolimus and AKT inhibitors [41, 42]. It was hypothesized that AKT affects the BCL2 protein and NF-κB pathway, although PI3K may also induce upregulation of the chemoresistance proteins, MDR1/Pgp, BCL2 and XIAP, while downregulating the expression of BAX and caspase 3. In tumour tissues from GC patients, which were examined in vitro, AKT activation and *PTEN* loss were associated with increased resistance to multiple chemotherapeutic agents (5-FU, doxorubicin, mitomycin C and cisplatin) [43]. Similarly, a combination of PI3K and AKT inhibitors with chemotherapy agents successfully attenuated chemotherapeutic resistance in a synergistic manner in GC cell lines [44, 45].

JAK2 is overexpressed in a subset of EBV-subtype GCs, and the JAK/STAT signalling pathway has been detected in several types of tumours, including GC [46, 47]. Following the activation of JAK2 by phosphorylation, STAT phosphoryla-

tion is induced and gene expression involved in cell proliferation, and apoptosis arrest is stimulated [48]. Therefore, JAK2 inhibitors may also represent a potential therapeutic treatment for GC. To date, the JAK pathway has been primarily a targeted strategy for myeloproliferative and inflammatory disorders and has only recently extended to solid tumours [49]. Regarding gastrointestinal malignancies, ruxolitinib, a JAK1 and JAK2 inhibitor, has demonstrated preliminary efficacy in combination with capecitabine in pancreatic adenocarcinoma and is currently under evaluation in colorectal cancer in combination with regorafenib [ClinicalTrials.gov identifier: NCI02119676] [50]. To the authors' knowledge, there are no trials ongoing in GC.

GC with MSI

According to the TCGA molecular classification, enrichment for microsatellite instability (MSI) characterizes a distinct molecular subgroup of GC. MSI occurs in approximately 15–30% of GCs and more frequently correlates with intestinal histotype, location in the distal part of the stomach, female gender and older age at diagnosis [10, 51, 52] (Fig. 10.2). MSI is a genetic alteration consisting of the expansion or contraction of regions of repetitive nucleotide sequences, called microsatellites. The alteration is triggered by a dysfunction of DNA mismatch repair (MMR) enzymes, caused by mutations in one of several different DNA mismatch repair genes (i.e. *MLH1* or *MSH2*). In a single cell, biallelic inactivation of *MMR* genes causes an increased mutation rate (genomic instability) due to the failure of DNA mismatch repair that usually occurs during normal DNA synthesis [53]. Defective DNA mismatch repair is the hallmark of Lynch syndrome. Different *MMR* genes are probably involved in MSI-high (MSI-H) sporadic gastric cancer without *MLH1* hypermethylation, which represents the main mechanism leading to MMR deficiency in MSI GC [54, 55]. In gastric cancer, 5-FU is frequently used, and information about sensitivity to this agent may be very useful. A meta-analysis of Zhu et al. [56] showed a 37% mortality risk reduction and improved median OS in patients with MSI-H compared with MSI-L (low) or microsatellite-stable (MSS) GC patients. The relationship between MMRd, MSI and survival has been examined in patients with resectable GC randomized to surgery alone or perioperative chemotherapy within the MRC MAGIC trial. MSI and *MLH1* deficiency was associated with a better outcome in patients treated with surgery alone, while it had a negative prognostic effect in those treated with chemotherapy [54]. Although MSI cases generally lack targetable amplifications, mutations in *PIK3CA*, *ERBB3*, *ERB22* and *EGFR* are noted [10, 52]; *BRAF* V600E mutations, commonly seen in MSI colorectal cancer, are absent in MSI GC [10]. However, the predictive role of these mutations in MSI GC population is uncertain.

Major histocompatibility complex class I gene alterations are common in this subtype. This, together with the increased number of tumour-specific neoantigens derived from hypermutated genes, suggests a potential additional role of immunotherapy for this category of tumours. Evidence of the activity of pembrolizumab in a subset of patients with MSI-positive colorectal cancer has recently been presented; the immune-related objective response and progression-free survival rates were 40% and 78%, respectively [57]. One potential challenge in developing suitable candidate therapies for patients with MSI- and EBV-type tumours is that, paradoxically, these patients are likely to have improved survival following surgery compared with patients with other subtypes. Both MSI and EBV positivity have been validated as favourable prognostic factors in resected GC and, therefore, may be present in lower proportions in the metastatic setting, with subsequent difficulty in identifying cases hindering the trial design [58, 59].

GC with CIN

The largest group, the CIN subtype, accounts for approximately 50% of GCs, and its most frequent location is the oesophagogastric junction (EGJ)/cardia, as established by TCGA study [10]. CIN molecular features include alterations in both the DNA copy number and structural abnormalities

in specific chromosomal regions. Those alterations could result in the gain or loss of entire chromosomes [60] (i.e. aneuploidy), nonreciprocal translocations, amplifications, deletion or the loss of one allele with the loss of heterozygosis. When CIN GC has an intestinal-type histology, it is associated with copy number gains of chromosomes 8q, 17q and 20q, whereas gains at 12q and 13q are more related to diffuse histology [61]. The final effect of the above-mentioned alterations is the loss or gain of function of oncogenes and tumour suppressor genes that may be efficaciously targeted by specific molecules [62]. Additionally, CIN subtype mutations in TP53 gene and receptor tyrosine kinases (RTKs) are frequently found as well as amplifications of cell cycle genes (Cyclin E1, Cyclin D1 and Cyclin-dependent kinase 6) and of the gene that encodes the ligand Vascular Endothelial Growth Factor A (VEGFA) [10, 63].

Furthermore, CIN displays amplification in oncogene pathways such as RTK/RAS/MAPK signalling, which includes HER2, BRAF, epidermal growth factor (EGFR), MET, FGFR2 and RAS [10, 64].

HER2

The proto-oncogene HER2 is a member of the EGF receptor tyrosine kinase family. The HER2 overexpression/amplification rate is different according to the site and histotype of GC: it is detected in more than 30% of tumours arising from the EGJ and in less than 20% of tumours in the gastric body; in addition, the intestinal and diffuse histotypes show rates of HER2 positivity of 34% and 6%, respectively [65]. Overexpression of HER2 has also been associated with HER2 amplification in 24% of CIN GC cases and subtypes other than CIN: in 12% of EBV cases and in 7% of MSI molecular subtypes [10]. Moreover, overexpression of HER2 has been associated with a poor prognosis and more aggressive disease. The established combination of chemotherapy and HER2-targeted therapy with trastuzumab had created a new standard of care for HER2-positive metastatic GC [33, 66, 67], as demonstrated in the trastuzumab for gastric cancer (ToGA) trial [66]. Following ToGA, several anti-HER2 agents were examined. The addition of pertuzumab (a monoclonal antibody blocking HER2/HER3 dimerization) to trastuzumab and docetaxel has already demonstrated a survival benefit in patients with breast cancer [68]. To evaluate this combination in GC, a phase III multicentre international clinical trial of pertuzumab or placebo in combination with trastuzumab and cisplatin-fluoropyrimidine regimen is ongoing. At this time, standard salvage treatment options for HER2-positive tumours are similar to those for HER2-negative disease; however, blockade of the HER2 pathway beyond trastuzumab progression is under investigation. The antibody-drug conjugate trastuzumab emtansine (T-DM1) has been evaluated in the second-line setting in a phase II clinical trial in previously treated patients with HER2-positive metastatic or advanced GC compared with docetaxel or paclitaxel. MM-111, a bispecific antibody fusion protein binding both HER2 and HER3 (the preferred dimerization partner of HER2), has been evaluated in a phase II clinical trial in combination with trastuzumab and paclitaxel [ClinicalTrials.gov identifier: NCT01774851]; however, this trial was terminated following inferior progression-free survival (PFS) results in the experimental arm. Lapatinib, a dual inhibitor of EGFR and HER2, has been examined as a first-line treatment in combination with capecitabine and oxaliplatin and second-line treatment in combination with paclitaxel [69, 70]. Both phase III studies failed to meet their primary endpoint (increased OS), and promising results were achieved. In the TRIO013/LOGiC trial, the primary endpoint of OS was not reached ($p = 0.35$), but patients of Asian ethnicity and patients younger than 60 years demonstrated a significant benefit in survival [69]. Similarly, in the TyTAN trial, patients with previously treated HER2 FISH-amplified GC were randomized to paclitaxel + lapatinib vs placebo, without having significantly longer OS ($p = 0.1044$). However, in patients with HER2 FISH amplification and 3+ score immunohistochemistry (IHC), a higher response rate was found [70]. Investment in dual inhibitors of the ERBB family continues; at a recent American Society of Medical Oncology (ASCO) Annual Meeting in 2015, an oral revers-

ible tyrosine kinase inhibitor of EGFR and HER2 named S-222611 demonstrated a 15% response rate (including one complete response) in HER2-positive GC [71].

The acquired resistance to HER2 inhibitors has also been studied in GC, exploring various molecular mechanisms underlying this phenomenon. Lee et al. [72] discovered several patterns of synchronous molecular alterations in the group of HER2 GC. In the paper by Zuo et al. [73], trastuzumab-resistant NCI-N87/TR cells were derived from the human gastric carcinoma cell line NCI-N87 with high HER2 expression, by stepwise exposure to increasing doses of trastuzumab. Activation of the downstream PI3K-AKT signalling pathway was one of the major mechanisms of resistance of NCI-N87/TR gastric cancer cells to trastuzumab, likely associated with PTEN gene downregulation and mutation, as well as with overactivity of the IGF-1R signalling pathway [73]. The most relevant finding of the study by Piro and colleagues [74] was that the inhibition of FGFR3 could be a potential strategy to modulate this resistance. IQ-domain GTPase-activating protein 1 (IQGAP1) is a multifunctional scaffold protein that interacts with diverse proteins to regulate cell adhesion and cell migration [75]. It was demonstrated in breast cancer cell lines that IQGAP1 plays an important role in HER-2 expression, phosphorylation and signalling [76], and its overexpression is correlated with trastuzumab-induced resistance and aggressive forms of gastric cancer [77]. Recently, Arienti et al. [75] revealed that high IQGAP1 expression leads to resistance to trastuzumab in GC; in addition, they found two new mutations of the HER2 gene that may be correlated with acquired resistance to the drug. Moreover, functional crosstalk between the receptor tyrosine kinase MET and HER family members has been reported in the context of the acquisition of aggressive phenotypes [78]. Hepatocyte growth factor (HGF)-mediated activation of MET may also cause resistance to lapatinib in HER2-amplified GC cell lines by stimulating downstream signalling [79]. De Silva et al. [80] confirmed in vitro that MET is likely to be a significant mechanism of lapatinib resistance in vivo.

EGFR

EGFR gene amplification is the second most frequent RTK alteration reported in the GC TCGA study and is demonstrated in 10% of CIN subtype tumours. Unfortunately, disappointing results from two large randomized phase III trials have discouraged further investigation of anti-EGFR agents in molecularly unselected populations [81, 82]. Panitumumab added to epirubicin, capecitabine and oxaliplatin as first-line treatment for metastatic or locally advanced oesophagogastric adenocarcinomas resulted in detrimental outcomes compared with chemotherapy alone [82]. A possible explanation for this outcome was hypothesized to be a reduction in the chemotherapy dose intensity due to overlapping toxicity and potentially negative interactions between anti-EGFR agents and oxaliplatin-based regimens. In the EXPAND trial [81], cetuximab, another anti-EGFR agent, did not lead to a survival benefit when added to the cisplatin-capecitabine regimen in previously untreated advanced junctional or gastric adenocarcinoma. Additionally, two anti-EGFR tyrosine kinase inhibitors, erlotinib and gefitinib, were ineffective in phase II and phase III trials compared with best supportive care in oesophageal and junctional cancers [83, 84].

MET

In an unselected GC case series, MET protein expression by IHC was identified in wide ranges, from 22% to 90% [85, 86], whereas MET amplification ranged between 2% and 10% and was confirmed in 8% of CIN subtype tumours in the TCGA series [10]. Both MET overexpression and MET amplification have been validated as negative prognostic factors in GC, and this pathway was, therefore, considered a valid target for pharmacologically specific therapies [10]. Unfortunately, both monoclonal antibodies and RTK inhibitors targeting the MET pathway failed to realize their own potential [87, 88]. Rilotumumab, a fully human monoclonal antibody targeting hepatocyte growth factor (HGF), a ligand of the MET receptor, was associated with significantly longer PFS and OS when added to epirubicin, cisplatin and capecitabine chemother-

apy in treatment-naïve molecularly unselected patients with advanced gastric or EGJ tumours in a multicentre phase II trial [89]. In patients selected for MET-positive expression/amplification, OS was longer in rilotumumab-treated patients compared with those on placebo (10.6 versus 5.7 months, respectively). However, the randomized phase III trial recently presented by Cunningham and colleagues in patients with MET-positive tumours by IHC was terminated prematurely due to an imbalanced number of deaths in the experimental arm [87]. Similarly, another anti-MET antibody, onartuzumab, showed no advantage in combination with mFOLFOX [88].

VEGFA Pathway

VEGFA is a member of the VEGF family, and it encodes a disulphide-linked homodimer that acts on endothelial cells and regulates vascular permeability, angiogenesis, vasculogenesis and endothelial cell growth, thereby promoting cell migration and inhibiting apoptosis.

VEGFA overexpression, reported in 54–90% of GC cases, is described as an early marker in the development of GC [90–92] and has been found to correlate with lymph node metastasis and poor prognosis. Other growth factors, VEGFC and VEGFD, are also overexpressed in 50–80% of GC cases, and high levels of expression correlate with lymphatic invasion [93, 94]. Interestingly, recurrent amplification of VEGFA has recently been reported to be a trait of the CIN subtype of GC, and this subgroup of cases may be a candidate for VEGF-targeting therapies [10].

Anti-angiogenesis therapies have been well-studied for cases of advanced-stage GC. For example, in the multinational, placebo-controlled phase III trial, Avastin in Gastric Cancer (AVAGAST), the efficacy of adding bevacizumab to an XP protocol for the first-line treatment of advanced-stage GC was examined. Unfortunately, AVAGAST did not accomplish its primary endpoint of extending the OS of patients with GC [95]. However, subgroup analyses demonstrated that significantly longer OS periods were achieved for patients from non-Asian regions [96]. Furthermore, in the RAINBOW trial, paclitaxel plus ramucirumab versus paclitaxel plus placebo were compared for the treatment of advanced, pretreated cases of GC. The results of this trial confirmed the survival advantage of ramucirumab plus paclitaxel for the treatment of GC in the non-Asian population [97]. The absence of a survival benefit in the RAINBOW and AVAGAST trials in the Asian subset population could be explained by various reasons: (i) the OS in Asian patients is always longer than that in the non-Asian population; (ii) patients from Asia had a better performance status; and (iii) the molecular differences between Asian and Western patients (i.e. different rates of the TCGA subgroups in the two ethnicities) could have affected the results.

FGFR

FGFR2 amplification is associated with tumour cell proliferation and survival of GC cell lines and is related to a poor prognosis. In TCGA classification, approximately 9% of CIN GC patients had FGFR2 gene amplification. Several drugs and studies targeting this mutation are ongoing [10]. A phase II randomized trial is evaluating the activity of AZD4547 (a FGFR 1–2 and 3 inhibitor) compared with paclitaxel in second-line treatment. Other ongoing trials are testing dovitinib in FGFR2-amplified GC patients or in combination with docetaxel [33].

KRAS and BRAF

KRAS mutation occurs in less than 5% of GC and is considered to have a negative prognostic impact in GC patients. KRAS activates critical pathways involved in carcinogenesis and tumour progression, including PI3K-Akt, RAF, MEK-extracellular signal-regulated kinase and NF-κB. However, no target therapies are currently approved for this alteration [98].

Genomically Stable (GS) GC

The GS subgroup includes all tumours that did not meet the criteria for the previously discussed three subtypes [10]. This subtype represents 20% of the TCGA samples and has been associated

with diffuse histology, earlier age at presentation (median: 59 years) and distal localization, and it occurs equally in males and females (Fig. 10.2). Several unique subtype-specific molecular changes have been described for GS tumours. The principal somatic genomic alterations observed in GS gastric tumours involve *CDH1*, *ARID1A* and *RHOA*. In addition, recurrent interchromosomal translocation (between *CLDN18* and *ARHGAP26*) implicated in cell motility was found in GS GC [10].

CDH1

The *CDH1* gene is located on chromosome 16q22.1 and encodes E-cadherin, which belongs to the cadherin superfamily of calcium-dependent cell adhesion molecules. Inactivating mutations in the *CDH1* gene are frequently found in gastric cancer, especially in hereditary diffuse gastric cancer [99], whereas CDH1 epigenetic promoter methylation is also frequently found in sporadic gastric cancer [100]. In the analysis of TCGA Research Network [10], *CDH1* somatic mutations were more frequent in the GS subtype (37% of cases). Li et al. [101] discovered that, in diffuse-type GC, *CDH1* mutation is associated with shortened OS, independent of disease stage.

ARID1A

Inactivating mutations of *ARID1A* were found in both GS and EBV-related GC [10]. The *ARID1A* gene, located in chromosome 1p35.3, encodes for adenine-thymine-rich interactive domain-containing protein 1A, which is involved in chromatin remodelling and regulating cellular processes, including DNA repair, differentiation and development [102]. As shown by Wang et al. [103], the loss of *ARID1A* expression was significantly correlated with tumour stage, grade and poor survival in GC patients.

RHOA

Rho GTPases are important intracellular signalling molecules that regulate cytoskeleton organization, cell cycle and cell motility. In cancer, Rho activity promotes metastasis by disrupting the epithelial layer, increasing motility and inducing degradation of the extracellular matrix [104]. *RHOA* mutations have been found to be strongly related to GC with a diffuse histotype [10].

Ripasudil, a selective inhibitor of Rho-associated coiled coil-containing protein kinase (ROCK), was approved in Japan in September 2014 for the treatment of glaucoma and ocular hypertension [105]. Accordingly, it is expected that newly developed drugs inhibiting the RhoA pathway will be evaluated in clinical trials for GC.

TCGA network analysis discovered a recurrent interchromosomal translocation between claudin 18 (*CLDN18*), a component of the tight junction adhesion structures [106], and Rho GTPase-activating protein 6 (*ARHGAP26*), resulting in the *CLDN18-ARHGAP26* fusion gene, which primarily occurs in GS GC [10]. ARHGAP26 is a GTPase-activating protein that facilitates the conversion of RHO GTPases to the GDP state and has been implicated in enhancing cellular motility. Yao et al. [107] showed that the expression of the *CLDN18-ARHGAP26* fusion gene resulted in the epithelial-mesenchymal transition of gastric epithelial cells and, therefore, in cell transformation and cancer development. A recent trial tested IMAB362, a chimeric IgG1 antibody against CLDN18.2 showing clinical activity in patients with 2+/3+ immunostaining [108]. The *CLDN18-ARHGAP* fusions were mutually exclusive with *RHOA* mutations; within the GS subtype, 30% of cases had either *RHOA* or *CLDN18-ARHGAP* alterations [10].

Patient-Derived Preclinical Models of GC

The lack of effective preclinical models of human tumours, reflecting the complexity and heterogeneity of cancer, has consistently limited the development of targeted drugs. Available models include cancer cell line in vitro and cell line xenograft mouse in vivo models, as well as organoids (Table 10.1). However, cell lines cannot replicate the heterogeneity of tumour cells or the relationship between the tumour and microenvironment.

Table 10.1 Patient-derived preclinical models of GC: advantages and disadvantages

	Cons	Pros
Cell line xenografts	Monodimensional No tumour microenvironment interaction Loss of architecture Genetic modifications	Rapid analysis of drug response Immortal cell lines allow an unlimited source of material Low cost, low complexity
PDX models	Limited source of material High failure rate of engraftment Long time for establishment Expensive Tissue must be rapidly processed	Reliable representation of tumour heterogeneity Includes microenvironment Can predict response to drugs
Organoids	No tumour microenvironment interaction	High level of architectural and physiological similarity to native tissue Intermediate cost, easy to handle Large-scale drug screening

Moreover, cell lines are usually established from aggressive tumours and are derived from a specific cell population; irreversible genomic alterations in the process of generating cancer cell lines have been observed [109–111]. For these reasons, this model failed to meet expectations in clinical trials, necessitating an alternative preclinical model to bypass these issues.

PDX models are xenograft mouse tumour models that are established by transplanting human tumour fragments into immunodeficient mice. The neoplastic tissue contains not only cancer cells but also the stroma, and this model can represent cancer heterogeneity. However, there are some inconveniences in the PDX models: the source of original material is limited, transplantation must be conducted rapidly and it is expensive and labour-intensive to establish and maintain the models [109]. A clearly important aspect is that PDX models of human GC constructed using subcutaneous or orthotopic implantation of surgical tissues or gastroscopic biopsies can reliably replicate the morphology and genetic alterations of native tumours [112–115]. Moreover, orthotopic implantation of GC tissue can lead to primary and metastatic tumour growth mimicking the progression of tumour stage, as seen in patients [112]. In one study using PDX models generated by subcutaneous implantation, CD44v8-10 was verified as a GC stem cell marker [116]. In another study, in vivo high-throughput screening using a 1 × 1 × 1 experimental design (a "one animal per model per treatment" approach) with PDX models assessed population responses to 62 treatments across 6 indications, including GC112; these latter data demonstrated the reproducibility and clinical translatability of PDX clinical trials by identifying associations between a genotype and a drug response and established mechanisms of resistance [117]. Similarly, based on the genomically defined GC PDX models, combination therapy of irinotecan with a BCL2L1-targeted drug was confirmed to effectively reduce the tumour size [118].

Organoids are miniature replicas of tissues cultured three-dimensionally in a semi-solid extracellular matrix and growth factor-enriched medium. Organoids sustain high levels of architectural and physiological similarity to native organ systems, superior to traditional two-dimensional homogeneous cell lines [119]. Additional advantages of organoids are that they are self-organizing, easy to handle, acceptable in cost, accessible to genetic engineering and amenable to large-scale drug screening with shorter turnaround times [120]. In a study using pluripotent stem cell-derived gastric organoids, *H. pylori* induced robust activation of c-Met by tyrosine phosphorylation and a twofold increase in epithelial cell proliferation. Cytotoxin-associated gene A played a pivotal role in this process, forming a complex with the c-Met receptor [121]. In another related study, gastric organoids exhibited dysplasia and readily generated adenocarcinomas in mice characterized by activating mutations in KRAS or loss of TP53 [122]. The potential metastatic role of TGFBR2 loss-of-function mutations was shown in CDH1–/–; TP53–/– murine

epithelial-mesenchymal organoids used to model hereditary GC, with short hairpin RNA knockdown of TGFBR2 [123]. A critical role of RHOA function in mediating anoikis in diffuse-type gastric carcinogenesis was confirmed in mouse intestinal organoids containing stably expressed RHOA mutations [124]. Thus, organoids constitute a robust model system that may facilitate personalized therapy development by enabling high-throughput drug screening to identify gene-drug associations and by testing specific individual responses to different therapeutic agents [116, 125].

Conclusions

The recent molecular research on GC has generated plentiful data that are currently not integrated into clinical practice.

However, they may be of help in the design of future clinical trials to personalize treatment in several ways: (i) by helping to identify the driving pathways of tumour growth, (ii) by discovering potential drugs targeting such pathways and (iii) by finding predictable mechanisms of resistance and strategies to overcome them.

It must be emphasized that each targetable molecular alteration/pathway is not specific to a distinct subtype of GC; therefore, molecular subgroups alone are not sufficient to assign a patient to a clinical trial. By contrast, molecular characterization of patients is useful to select a small population to be screened for protocol-eligible molecular aberrations. To select the most appropriate therapies for patients with advanced-stage GC, the implementation of GC research and clinical trials in which patients can be classified based on molecular characteristics or molecular subtypes is required.

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Part V

Future Medicine in Gastric Cancer

11 Noncoding RNA in Gastric Cancer with Potential Prognostic and Predictive Role

Federica Rao, Flavio Rizzolio, Clara Rizzardi, Tiziana Perin, and Vincenzo Canzonieri

Introduction

GC is one of the most frequent malignant tumors; every year in the world, there are 723,000 cancer-related deaths caused by GC according to the World Health Organization (WHO). It is the fifth most common cancer in the world and the third cause of death among cancer pathologies [1]. Due to the lack of specific diagnostic markers, most patients with GC do not receive an appropriate diagnosis and treatment; this leads to a progression of the pathological state with development of metastases [2]. Previous studies have hypothesized that GC is a genetic disease involving multi-step changes in the genome [3]. However, the human genome contains nearly 20,000 protein-coding genes, but they represent less than 2% of the whole genome [4]. In contrast, according to the Encyclopedia of DNA Elements (ENCODE) project, more than 80% of functional DNA elements in the human genome do not code for proteins [5]. A large part of these functional DNA elements is represented by ncRNAs [6].

In the last years, several studies have shown that ncRNAs play a significant role in different cellular and physiological processes including gene regulation, genomic imprinting, chromatin packaging, dosage compensation, cell differentiation, and embryonic development [6, 7]. Accordingly, the dysregulation of ncRNAs, as pivotal modulators of gene expression, has been documented in different human complex diseases including cancer [8]. In fact, they are able to influence different mechanisms in cancer cells,

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© Springer Nature Switzerland AG 2019
V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_11

such as proliferation, apoptosis, invasion, and metastasis as well as neoangiogenesis [9]. Expression profiling studies on ncRNAs in a variety of cancer types have revealed a broad range of lncRNAs with aberrant expression [10]. Moreover, it has been shown that ncRNAs are promising candidate prognostic biomarkers for GC detection and potential therapeutic targets. Several ncRNAs could be secreted into body fluids, suggesting that tumor cells may change their extracellular environments through RNA-based, hormone-like mechanisms [11].

In this chapter, we discussed the different roles of ncRNAs in GC and the possible diagnostic, prognostic, and therapeutic applications.

ncRNAs

ncRNAs refer to a class of RNAs with no protein-coding function that are widely expressed in organisms [12]. ncRNAs can be divided into two groups: housekeeping ncRNAs and regulatory ncRNAs. The latter can further be divided into three types, according to their length: (1) short ncRNAs, including miRNAs, small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs), (2) mid-size ncRNAs, and (3) lncRNAs [13–15].

Short ncRNAs are shorter than 50 nucleotides (nt), mid-size ncRNAs have a length between 50 and 200 nt, and lncRNAs are longer than 200 nt [16].

Currently, numerous studies have found that miRNAs and lncRNAs play important roles in GC progression.

Table 11.1 summarizes the characteristics of different groups of ncRNAs.

miRNAs in GC

miRNAs are a class of small ncRNAs of approximately 18–24 nt. Genes encoding miRNAs could be single copy, multiple copies, or clusters; other forms exist in the region of protein-coding genes, including introns. They are highly conserved sequences and have temporal and tissue specificity [17].

Although miRNAs do not code for proteins, they have an important role in the regulation of gene expression at the posttranscriptional level. Through complete or incomplete complementary binding to the 3′-untranslated regions (3′-UTRs) of target mRNAs, miRNAs promote the degradation of targeted-mRNA or their translational suppression. As a consequence of this process, which involves the recruitment of a number of other proteins, miRNAs are able to regulate negatively the expression of target genes [18, 19].

One miRNA interacts with several different mRNAs in different regions. A mRNA could also combine with several miRNAs on the basis of complete or incomplete sequence complementarity.

The synthesis of miRNA involves the production of a primary transcript (pri-miRNA) from genomic DNA by polymerase II within the nucleus. Then, the pri-miRNA is cut by the Drosha enzyme of RNase 3 endonuclease enzyme family into hairpin precursors of miRNA (pre-miRNA), which are approximately 70 nt [20]. Finally, the synergistic effect of Ran-GTP and transporter protein Exportin 5 transports pre-miRNA out of the nucleus, and the enzyme Dicer cuts it to produce the approximately 22 nt mature miRNA [21]. At this point, the synthesized miRNA is ready to exert its function.

Through the latest approaches of microarray technology, bioinformatics, and other genetics methods, the ectopic expression of miRNAs in GC has been found to be closely related to different steps of cancer initiation and progression including metastasis. By upregulation of the expression of oncogenes or downregulation of the expression of tumor suppressor genes, miRNAs play an important role in the regulation of cancer-related genes. A first example can be given by miRNA-106b-25. Petrocca et al. reported that an abnormal regulation of the transcription factor E2F1 and transforming growth factor-β (TGF-β) plays a critical role in gastric carcinogenesis. E2F1 activates its own promoter and miR-106b-25 cluster expression simultaneously with its host gene, Mcm7. Furthermore, the TGF-β tumor suppressor pathway was impaired by overexpression of the miR-106b-25 cluster, but also the expres-

Table 11.1 Classification of human genomic ncRNAs

RNA type	Symbol	Length (nt)	Function	References
Housekeeping ncRNAs				
Transfer RNAs	tRNA	70–80	Connect amino acids with mRNA [1]	(1) Lodish H, Berk A, Zipursky SL, et al. Molecular cell biology. 4th ed. New York: W. H. Freeman; 2000
Ribosomal RNAs	rRNA	121–5070	Component of ribosomes [1]	
Small nuclear RNAs	snRNA	≈ 150	Assemble with proteins into spliceosomes to remove introns during mRNA processing [2]	(2) Valadkhan S, Gunawardane LS. Role of small nuclear RNAs in eukaryotic gene expression. Essays in Biochemistry. May 03, 2013, 5479–90. https://doi.org/10.1042/bse0540079
Small nucleolar RNAs	snoRNA	70–200	Guide modifications of other ncRNAs, alternative splicing; or function as miRNA [3]	(3) Scott MS, Ono M. From snoRNA to miRNA: dual function regulatory non-coding RNAs. Biochimie. 2011;93(11):1987–92. https://doi.org/10.1016/j.biochi.2011.05.026
Telomerase RNAs	TERC	451	Provide template for de novo synthesis of telomeric DNA [4]	(4) Theimer CA, Feigon J. Structure and function of telomerase RNA. Curr Opin Struct Biol. 2006;16(3):307–18. https://doi.org/10.1016/j.sbi.2006.05.005
Ribonuclease P	RPPH1	341	RNA component of ribonuclease P [5]	(5) Altman S, Ribonuclease P. Philos Trans R Soc Lond B Biol Sci. 2011;366(1580):2936–41. https://doi.org/10.1098/rstb.2011.0142
Regulatory ncRNA				
Small interfering RNAs	siRNA	21–22	Silencing genes in a sequence-specific manner [6]	(6) Dana H, Chalbatani GM, Gharagouzlo E. Molecular mechanisms and biological functions of siRNA. Int J Biomed Sci. 2017;13(2):48–57. Available on: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5542916/#__ffn_sectitle
MicroRNAs	miRNA	20–23	Regulating gene expression [7]	(7) MacFarlane L-A, Murphy PR. MicroRNA: Biogenesis, function and role in cancer. Curr Genomics. 2010;11(7):537–61. https://doi.org/10.2174/138920210793175895
Piwi-interacting RNAs	piRNA	25–33	Repress transposons and maintain germline genome integrity [8]	(8) Iwasaki YW, Siomi MC, Siomi H. PIWI-Interacting RNA: its biogenesis and functions. Ann Rev Biochem. 2015;84:405–33. d https://doi.org/10.1146/annurev-biochem-060614-034258
Promoter-associated RNAs	paRNA	<200	Regulating gene expression [9]	(9) Yan BX, Ma JX. Promoter-associated RNAs and promoter-targeted RNAs. Cell Mol Life Sci. 2012;69(17):2833–42. https://doi.org/10.1007/s00018-012-0953-1
Long noncoding RNAs	lncRNA	>200	Various [10]	(10) Ahmad Bhat S, Mudasir Ahmad S, et al. Long non-coding RNAs: mechanism of action and functional utility, Non-coding RNA Res. 2016;1(1):43–50. https://doi.org/10.1016/j.ncrna.2016.11.002

sion of the factors CDKN1A (p21Waf1/Cip1) and BCL2L11 (Bim) is altered. Finally, CDKN1A and BCL2L11 disrupted the G1/S checkpoint and conferred resistance to TGF-β-dependent apoptosis, respectively (Fig. 11.1) [22].

A different example can be given by miRNA-9, which is downregulated in GC. A direct target of the miRNA-9 molecule is the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB1). A study conducted by Wan et al. has

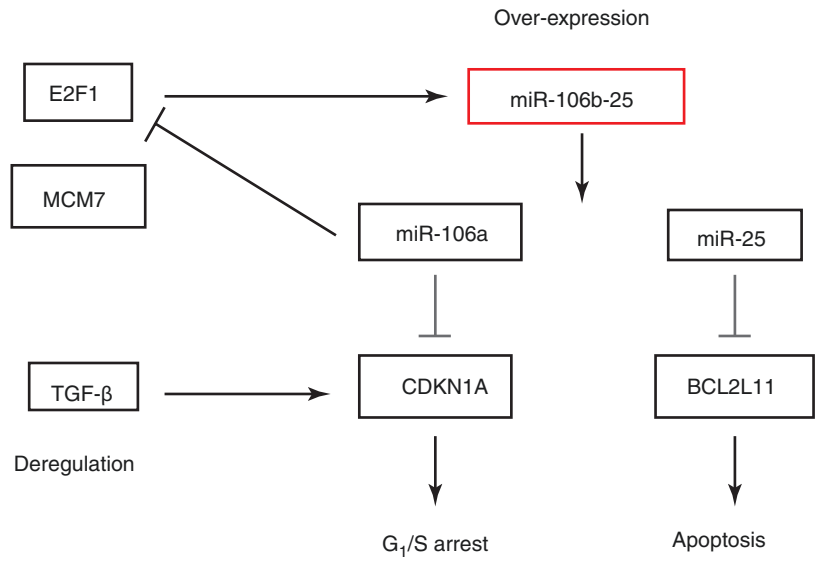

Fig. 11.1 Functions of microRNA-106b-25. miRNA-106b-25 interferes with the expression of CDKN1 and BCL2L11. The interaction of miRNA-106b-25 with E2F1 and transforming growth factor-β (TGF-β) affects the cell cycle and apoptosis

shown that cell growth and proliferation were significantly inhibited by overexpression of miR-9 that not only inversely regulates endogenous NF-κB1 protein expression but also reduces endogenous NF-κB1 mRNA levels [23].

In a more recent study by Tae-Su Han and his colleagues, several GC-specific miRNAs have been identified through comprehensive miRNA profiling using a next-generation sequencing (NGS) platform. It was discovered that miR-29c expression was downregulated in GC tissues. Moreover, a tumor suppressor role was identified for miR-29c, which regulates its downstream target gene, ITGB1, in GC. The suppression of miR-29c is an early event in gastric carcinogenesis [24].

Chemotherapeutic resistance is a big problem that has not yet been solved in GC treatment. Multiple reports have suggested that miRNAs are associated with the sensitivity of GC cell lines to chemotherapy. For example, miR-375 was conspicuously downregulated in cisplatin (DDP)-resistant cells compared with the DDP-sensitive human GC cell line. Western blot analyses showed that upregulation of miR-375 increased GC cell sensitivity to DDP treatment by targeting ERBB2 and phosphorylated Akt. The antiproliferative and apoptosis-inducing effects of DDP could be reversed by reducing the level of miR-375 [25].

Many other miRNAs, like miR-448, miR-15a, and miR-485-5p, were found to suppress proliferation, invasion, or migration in GC cell lines via their target genes such as IGF1R, Bmi1, and Flot1, respectively [26–28].

Other miRNAs, such as miR-1290 and miR-543, could promote gastric tumor cell proliferation or metastasis by targeting their downstream genes FOXA1 and SIRT1 [29, 30].

lncRNA in GC

lncRNAs are the largest class of ncRNAs ranging from 200 nt to several kilobases in length. It is possible to classify them into different groups based on their genomic localization, mode of action, and function. On the base of their genomic location, five main types can be distinguished: antisense, intronic, intergenic, bidirectional, and sense-overlapping lncRNAs. Based on their mode of action on DNA sequences, there are two classes of lncRNAs: cis-acting lncRNAs and trans-acting lncRNAs. Functionally, lncRNAs may be grouped into four types: signaling, decoy, guide, and scaffold (Fig. 11.2) [6, 31]. lncRNAs take part in various cellular and physiological processes such as gene regulation, genomic imprinting, chromatin packaging, dosage compensation, cell differentia-

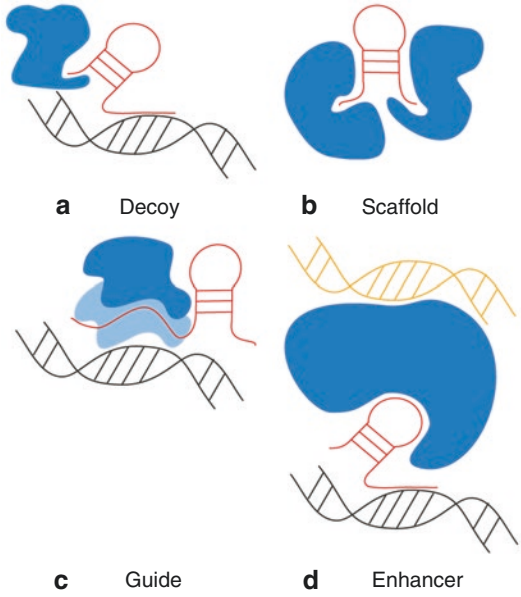

Fig. 11.2 Four types of lncRNA mechanisms: (**a**) The lncRNAs can act as decoys, titrating away DNA-binding proteins (e.g., transcription factors); (**b**) lncRNAs may act as scaffolds to bring two or more proteins to spatial proximity or into a complex; (**c**) lncRNAs may act as guides to recruit proteins to DNA (e.g., chromatin modification enzymes); and (**d**) lncRNA guidance can also be exerted through chromosome looping in an enhancer-like model in cis. lncRNA (red), DNA (black), section of DNA loop (yellow), DNA-binding proteins (blue). (Source: Luka Bolha et al. [31], Article ID 7243968, 14 pages, Fig. 1, https://doi.org/10.1155/2017/7243968, an open access article distributed under the Creative Commons Attribution License)

tion, and embryonic development [6]. Being pivotal regulators of gene expression, alterations of lncRNA can be found in different diseases including cancer. In fact, they influence the main mechanisms related to cancer including proliferation, apoptosis, invasion, and metastasis as well as neoangiogenesis [9].

lncRNAs expression profiling in a variety of cancer types has revealed a broad range of lncRNAs with aberrant expression.

lncRNA Upregulated in GC

Ak058003 is transcribed from its locus at chromosome 10q22, and it has a length of 1197 base pairs (bp). Wang et al. have discovered that the expression of Ak058003 increased during hypoxia. Moreover, this lncRNA is upregulated in GC, and its elevated level is accompanied by an increase in cell migration in vivo and in vitro. Furthermore, this lncRNA targets the γ-synuclein (SNCG), a prometastatic oncogene. Increased AK058003 expression decreases SNCG promoter methylation and consequently upregulates the expression of this oncogene, which promotes hypoxia-induced GC cell metastasis [32].

ANRIL is transcribed in an antisense direction by a locus located on 9p21.3 [33]. It has been shown that ANRIL can act as a scaffold or guide to chromatin [34]. According to recent studies, ANRIL binds to PRC2 and epigenetically represses the expression of miR-99a and miR-449a. In GC, the levels of ANRIL and miR-99a/miR-449a are inversely related so that the expression of these two miRNAs is decreased and the level of ANRIL expression is high in GC samples. This leads to a high tumor-node-metastasis (TNM) stage and tumor size [35].

BANCR The BRAF-activated noncoding RNA (BANCR) gene is located on 9q21.1 and contains four exons. It encodes a lncRNA with a length of 693 bp. BANCR expression is elevated in many GC tissues and cell lines. It has been assessed that this lncRNA influences GC cell growth and apoptosis through regulating NF-κB1 expression via miR-9. Upregulation of BANCR contributes to a decline in NF-κB1 expression that leads to an increase in cell numbers and a decrease in apoptosis in GC cells [36]. Several studies have shown that overexpression of BANCR in GC tissues is correlated with clinical stage, lymph node, and distant metastases [37].

CCAT1 Colon cancer-associated transcript 1 (CCAT1) is 2628 nt long, and its gene is located at 8q24 [38]. CCAT1 is overexpressed in some GC tissues with a significant correlation with primary tumor growth, lymph node, and distant metastases. c-Myc oncogene physically interacts with E-box element in the CCAT1 promoter and increases its expression. In vitro, CCAT1 regulates cell proliferation and migration [39]. Other studies have demonstrated that CCAT1 activates the ERK/MAPK pathway and suppresses cell cycle arrest and apoptosis.

GACAT3 Located at 2p24, GACAT3 encodes a lncRNA of 1096 nt in length. It was observed that it is upregulated in GC tissues and this upregulation is positively correlated with TNM stages, tumor size, and distant metastasis [40].

H19 As a maternally imprinted gene, H19 is located on 11p15.5. H19 plays an important role during embryogenesis, and its expression is low in most adult tissues except for cardiac and skeletal muscles [41, 42]. It is associated with p53 protein, and reciprocally, p53 protein has repressing effects on H19 levels [43, 44]. H19 gene contains a 23 nt RNA, miR-675 [45]. It has been shown that H19 works via its miR-675 product to silence the transcription factor RUNX1, a tumor suppressor in GC, in turn inducing cell proliferation [41]. The amount of H19 and miR-675 is increased in GC tissues with a significant correlation with lymph node metastases and clinical stage [46]. H19 and miR-675 have different targets, but they both function as oncogenes to increase proliferation, migration, invasion, and metastases in human GC [47].

HOTAIR HOX transcript antisense RNA (HOTAIR) is transcribed from 12q13.13 and plays an important role in GC progression [48]; for this reason it is one of the most studied lncRNAs. HOTAIR is expressed from the HOXC locus, and its length is of 2158 nt [49]. Functioning as a scaffold, HOTAIR is involved in epigenetic silencing. It directs polycomb repression complex 2 (PRC2) to trimethylate histone H3 lysine-27 of specific HOXD genes and thus repressing their expression.

It is believed that HOTAIR can promote metastasis through this pathway by inhibiting certain metastasis suppressor genes [50]. It has been demonstrated that HOTAIR expression is markedly raised in GC tissues, which is associated with poor prognosis, higher TNM stage, perineural invasion, larger tumor size, and lymph node and distant metastases [49, 51].

MALAT1 Encoded at chromosome 11q13 with 8000 nt in length, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a lncRNA [52, 53]. It was observed that MALAT1 is overexpressed in GC tissues, which correlates with peritoneal metastasis in patients [54]. Furthermore, MALAT1 increases cellular proliferation by regulating alternative splicing factor 1 (ASF1) and pre-mRNA-splicing factor (SF2) (SF2/ASF1) [55]. These proteins are pivotal players in inflammatory disorders and also in cancer [56].

PVT1 Plasmacytoma variant translocation 1 gene (PVT1) is located on human 8q24, 57 kb downstream of c-Myc [57]. The 8q24 region with both genes is involved in a variety of cancer types.

It has been reported that this lncRNA has a role in the suppression of apoptotic genes in different types of cancer. Upregulation of PVT1 is essential for the increased level of c-Myc in cancer cells [58]. PVT1 expression is elevated in GC tissues as well. Furthermore, PVT1 may be involved in the silencing process of *CDKN2B/p15* and *CDKN2A/p16* genes through its association with EZH2 during the progression of GC [59]. Its overexpression is linked to lymph node metastases [57].

UCA1 Urothelial carcinoma associated 1 (UCA1) is located on 19p13.12, and it contains three exons [60]. UCA1 presents higher expression in GC tissues and cell lines. The expression was associated with tumor size, worse differentiation, invasion depth, and TNM stages. Analyses conducted in GC have reported that excessive amount of UCA1 correlates with poor overall survival and disease-free survival in patients [61].

lncRNA Downregulated in GC

AA174084 is a lncRNA downregulated in GC tissues compared with adjacent normal tissues. Studies conducted on samples of gastric juice in patients with gastric ulcer, chronic atrophic gastritis, or GC have shown that levels of this lncRNA were highest in GC patients, suggesting its potential value as a GC biomarker. AA174084 expression levels in GC tissues were associated with age, Borrmann type, and perineural invasion. Expression in gastric juice was associated with tumor size, tumor stage, Lauren type, and CEA levels. Overall, the current data show that the AA174084 level in gastric juice may be used as a screening biomarker for detecting GC at early stages [62].

FENDRR FOXF1 adjacent noncoding developmental regulatory RNA (FENDRR) is located on 16q24.1 and contains seven exons. Through binding to PRC2 and/or TrxG/MLL complexes, FENDRR lncRNA regulates histone methylation and chromatin structure [63]. Furthermore, FENDRR is diminished in GC tissues and cell lines, which correlate with depth of invasion, advanced tumor stage, and lymphatic metastasis.

FER1L4 Fer-1-like protein 4 (FER1L4) is located at 20q11. Its expression is reduced in GC tissues, and it is correlated with histological grade, tumor size, severity of invasion, vessel or nerve invasion, and lymph node and distant metastases [64]. FER1L4 is one of the targets of miR-106a-5p. Low quantity of this lncRNA increases the amount of free miR-106a-5p, making it more available for its targets such as the retinoblastoma gene, *RB1* [65].

GACAT2 Gastric cancer-associated transcript 2 (GACAT2) is encoded at 18p11, and it has a length of 818 nt. GACAT2 is markedly decreased in GC tissues and cell lines, which is associated with distal metastasis and neural and blood vessel invasion in GC tissues [66].

MEG3 Maternally expressed gene 3 (MEG3) is a tumor suppressor lncRNA transcribed from an imprinted gene cluster at 14q32, with a length of 1700 nt [67]. It has been demonstrated a significant decrease of MEG3 levels in GC tissues, and this was linked with TNM stage, tumor size, depth of invasion, and shorter overall survival time in GC patients [68].

MT1JP Metallothionein 1 J gene is located on 16q13. It has considerably lower expression in GC tissue samples than in matched normal tissues. Zhongchuan et al. have demonstrated that MT1JP is necessary for maintaining the normal life activities of cells and played a critical function as a tumor suppressor. lncRNA MT1JP is involved in many steps of tumor progression, including cell proliferation, migration, and invasion. For this reason, it may be a potential diagnostic marker and could have a potential therapeutic value in the prevention of GC [2].

ncRuPAR Noncoding RNA upstream of the PAR-1 (ncRuPAR) [69] increases the expression of protease activator-1 (PAR1) during embryonic growth. The study conducted by Liu et al. reports that it works as a tumor suppressor in cancer.

Its gene is located on human 5q13. Decreased expression of this lncRNA in GC samples was inversely correlated with the amount of PAR-1. Its level was negatively associated with tumor size, tumor invasion depth, lymph node, and distant metastases [70].

TUSC7 Tumor suppressor candidate 7 (TUSC7) is located on 3q13.31 and contains four exons. Some studies have reported that TUSC7 is downregulated in GC tissues contributing to an augmentation in cell growth. In addition, p53 is a regulator of TUSC7 in GC, and *TP53* mutations or deletions are the likely cause of TUSC7 downregulation. Furthermore, TUSC7 negatively regulates the level of miR-23b, which promotes cell growth in GC samples [71].

miRNA as Biomarker in GC

Numerous miRNAs are aberrantly expressed in the plasma and serum of GC patients [72–74]. For example, miR223, miR-233, miR-378, miR-421, miR-451, miR-4865p, and miR-199-3p are overexpressed in sera of GC patients [75–78]. Wang et al. found that miR-233 was overexpressed in GC patient sera, and its level was positively associated with tumor differentiation grade, TNM stage, tumor size, and metastasis status [75].

Wu et al. found that miR-421 was overexpressed in 90 cases of GC patient sera compared to 90 controls. The high expression of miR-421 in cancer cells acts as a biomarker for GC circulating tumor cells, which may be used for early diagnosis for gastric metastasis [76]. Furthermore, in vivo and in vitro experiments demonstrated that the onco-miR-421 promotes tumor proliferation, invasion, and metastasis but had no significant association with the clinic-pathological features [79, 80].

In contrast, the expression of miRNAs such as let-7a, miR-375, miR-20a-5p, and miR-320 was relatively reduced in GC patient sera [81, 82]. A study demonstrated that let-7a exhibited rela-

tively low expression in plasma of GC patients compared with healthy controls, whereas the expression of miR-17-5p, miR-106a, miR-106b, and miR-21 was significantly elevated in GC plasma [83]. Other studies demonstrated that miR375 was suppressed in GC. Overexpression of miR-375 suppresses GC progression by targeting p53, JAK2, ERBB2, and STAT3 [84, 85]. These studies indicate that miRNA could be useful diagnostic biomarkers. However, large-scale clinical research is needed to demonstrate that miRNA can serve as a diagnostic biomarker for GC.

Several studies have demonstrated that miRNAs could be used not only as biomarkers but also as potential therapeutic targets for cancer. miRNA-based drugs that act by suppressing miRNAs or inhibit the onco-miRNAs can inhibit tumor progression by suppressing the relative signal pathway [86, 87]. For example, miR-34 is one of the most characterized tumor suppressor miRNAs in a variety of tumors including GC. In literature, it is reported that it is lost or expressed at minimum levels in numerous tumor tissues, and the reintroduction of miR-34 mimics was found to inhibit cancer cell growth both in vitro and in vivo. Therefore, miR-34a has proved to be a tumor suppressor in cancer cells and an ideal therapeutic tool to reduce metastasis, chemoresistance, and tumor recurrence [88–90].

However, some problems should be considered; as one miRNA can target multiple genes and signaling, the off-target effect is not easily predictable. Thus, miRNA therapy needs more detailed studies [91].

lncRNA as Biomarkers in GC

In recent years, detection of cancer-associated lncRNAs in body fluids of cancer patients has proven itself as a valuable method to effectively diagnose cancer. Cancer diagnosis and prognosis through the use of circulating lncRNAs are preferred when compared to classical biopsies of tumor tissues, because of their noninvasiveness and great potential for routine applications in clinical practice.

Among main advantages of lncRNAs, which make them suitable as cancer diagnostic and prognostic biomarkers, is their high stability while circulating in body fluids, especially when included in exosomes or apoptotic bodies [92]. It has been shown that lncRNAs are able to resist the multiple ribonucleases in body fluids [93]. In addition, lncRNA deregulation in primary tumor tissues is clearly mirrored in various bodily fluids, including whole blood, plasma, urine, saliva, and gastric juice [94, 95]. These characteristics make the lncRNAs of potential prognostic and predictive biomarkers for GC, easy to take and evaluate, bringing great benefits to patients compared to a classic tissue biopsy [96].

The detection of circulating lncRNAs could represent an excellent method in the evaluation of cancer to distinguish tumor patients from healthy people at early stages with both high sensitivity and specificity. In addition, the prognosis of tumor patients and the risk of tumor metastasis and recurrence after surgery could be assessed [93]. Good results have been obtained from the diagnostic performances of lncRNAs BANCR, H19, CCAT, and AA174084 evaluated in body fluid samples (e.g., plasma and gastric juice) of GC patients. These lncRNAs had the ability to differentiate GC patients from healthy individuals and to effectively detect different stages of GC (from early to metastatic cancer forms). However, despite their overall positive diagnostic performances, similar to those obtained by several conventional cancer biomarkers, false-positive and false-negative detections were observed [95, 97, 98].

Stability of lncRNAs in body fluids of tumor patients has not been thoroughly explored. Studies revealed that some lncRNAs remained stable in plasma under extreme conditions, such as several freeze-thawed cycles and prolonged incubation at elevated temperatures [99]. So far, three mechanisms have been identified by which lncRNAs are released into body fluids. First, extracellular RNAs may package themselves into specific membrane vesicles, such as exosomes and microvesicles, in order to be secreted and resist to RNase activity. Different studies revealed that exosomes most frequently protect plasma

lncRNAs [100–103]. Second, extracellular RNAs can be actively released by tumor tissues and cells [104]. Third, extracellular RNAs may encapsulate themselves into high-density lipoprotein (HDL) or apoptotic bodies or be associated with protein complexes, for example, Argonaute (Ago)-miRNA complex and nucleophosmin 1 (NPM1)-miRNA complex [105, 106]. However, despite many performed studies, secretion and transport mechanisms of lncRNAs to the circulation system remain yet poorly understood.

In order to introduce circulating lncRNAs into clinical practice, further studies and improvements should be performed regarding the standardization of sample preparation protocols and the extraction methods [93].

Conclusion

In recent years, the role of ncRNAs in GC has been clarified. Multiple studies have already demonstrated the potential clinical applications of several ncRNAs in GC diagnosis and prognosis. Circulating ncRNAs are regarded as an emerging biomarker for GC, but the applications of circulating ncRNAs need to be further investigated because of the interactions between ncRNAs and GC that are very complex.

Among these, several ncRNAs are promising neoplastic biomarkers to be detected in the patient's body fluids, including miR-34, H19, HOTAIR, MALAT1, UCA1, and AA174084. For many of these ncRNAs, it has been proven that they could be used in clinical practice as diagnostic and prognostic GC biomarkers. ncRNA research will likely take a big step forward with the identification of more molecules in the next years.

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12 Immunomodulation and Immunotherapy for Gastric Cancer

Riccardo Dolcetti and Valli De Re

Introduction

Although surgery is a curative treatment for early-stage gastric cancer (GC), the median overall survival for patients diagnosed in a metastatic stage is less than 1 year [1]. New treatment options are therefore urgently needed. Recently, immunotherapy has emerged as one of the most promising strategies in cancer treatment, with outstanding results in several tumor types [2–4]. The clinical successes of immune checkpoint inhibitors have revolutionized cancer treatment clearly indicating that targeting the host immune system rather than the tumor may be more effective than conventional therapies. Although encouraging, the results so far obtained in GC patients are however still unsatisfactory, and the majority of novel immunotherapies in this setting are still in early-phase clinical investigation [5, 6]. Several complex factors are limiting the development of effective immunotherapeutic strategies for GC, including the heterogeneous immunogenicity among and within tumor subtypes and the different and still poorly defined immunosuppressive mechanisms that may hamper the effective control of the tumor by host immune cells. A deeper genetic and immunologic characterization of GC is required to allow for a more precise identification of patients who could benefit from modalities of immune intervention, as monotherapy or more likely within combination schedules. Here we will highlight the immunologic characteristics of different GC subsets, with particular focus on the tumor microenvironment, as a potential basis to improve tailoring of (immune) therapies. We will also review the state of the art of the various strategies of immunotherapy and immunomodulation investigated in the preclinical and clinical settings of GC.

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Antitumor Immune Responses

The critical role of host immunity in controlling cancer is now well recognized. Available evidence supports the concept that our immune system is able to prevent cancer development through a process termed immune surveillance [7]. Dying cancer cells may express and release tumor-specific and tumor-associated antigens that can be taken up and processed by tissue-resident dendritic

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_12

cells, which then maturate in antigen-presenting cells in the presence of an appropriate microenvironment, usually enriched in activator molecules, the so-called danger-associated molecular patterns (DAMPs) [7]. Induction of effective anticancer immunity requires that mature antigen-presenting cells efficiently present tumor antigens in the form of peptides to $CD8^+$ T lymphocytes through major histocompatibility complex (MHC) class I molecules and to $CD4^+$ T lymphocytes through MHC class II molecules. The strongest tumor antigens are those provided by nonself or mutated proteins, such as those encoded by viruses or generated by somatic mutations occurring in genes expressed by tumor cells. For an efficient activation of the $CD8^+$ T cells, both antigen presentation (first signal) and the presence of costimulatory molecules (second signal) are needed [7]. After activation, T lymphocytes proliferate and infiltrate the tumor bed; promote the recruitment of other immune cells, including natural killer (NK) cells and M1 macrophages; and directly kill cancer cells through the release of cytokines, perforin, and granzymes [7]. Of relevance in the light of clinical application of immunotherapeutic strategies is the notion that not all conventional cytotoxic chemotherapeutic drugs are immunosuppressive. Recent evidence clearly indicates that certain commonly used drugs, including doxorubicin, mitoxantrone, bortezomib, oxaliplatin, and cyclophosphamide, can kill tumor cells also by an immunogenic cell death pathway, which activates robust innate and adaptive antitumor immune responses [8]. The immunogenic cell death is the consequence of the activation of adaptive responses in dying cells, which ultimately result in the exposure or secretion of immunostimulatory molecules commonly referred to as "damage-associated molecular patterns" [8]. Radiotherapy is also capable of rendering tumor cells immunogenic by modifying their phenotype and the surrounding microenvironment [9]. After treatment, danger signals are locally released resulting in maturation of dendritic cells and priming of cytotoxic T cells as well as in activation of NK cells. Despite being a local therapy characterized by an impressively high degree of spatial accuracy, radiotherapy can elicit systemic immune effects, which occasionally lead to regression and rejection of non-irradiated, distant tumor lesions, the so-called abscopal effect [10]. On these grounds, the present challenge is to better understand the potential immunomodulatory properties of currently used chemo- or radiotherapeutic regimens, in order to maximize the efficacy of their combination with immunotherapeutic strategies.

Tumor Microenvironment and Immunogenicity of GC

Immunogenic Subtypes of GC

Recently, the Cancer Genome Atlas (TGCA) classified GC into four main molecularly defined subgroups: (1) Epstein-Barr virus (EBV)-positive GCs (~9% of all GC), which frequently carry PIK3CA mutations, PD-L1/PD-L2 overexpression, and extreme DNA hypermethylation; (2) microsatellite instability (MSI) tumors (15–30% of all GC), which are frequently hypermutated; (3) chromosomal instability (CIN) tumors (50%, mainly junctional), which show a high rate of copy number variations, TP53 mutations, and receptor tyrosine kinase-Ras activation; and (4) genomically stable (GS) GCs (20%), which show altered motility and mutations in adhesion molecules [11]. These findings stimulated a great interest to tailor therapeutic approaches according to the features of each GC subset. This may be particularly relevant for immunotherapeutic purposes, considering the different level of immunogenicity shown by the four TGCA subsets.

A meta-analysis demonstrated that patients with EBV-associated GC have a better prognosis as compared to those with an EBV-unrelated GC [12]. Although the underlying mechanisms are not clear, the extensive lymphocyte infiltration, particularly of $CD8^+$ T cells, which characterizes this GC subset suggests that antitumor immune responses triggered by viral antigens may have a role in determining a better clinical outcome [13, 14]. In EBV^+ GC, genes involved in cytokine/chemokine pathways are frequently deregulated [15], and programmed cell death ligand 1 (PD-L1) expression is markedly increased via multiple mechanisms [16].

MSI is characterized by alterations in length within short repeated DNA sequences (microsatellites), which are the consequence of inactivating mutations or epigenetic silencing of DNA mismatch repair genes (e.g., *MSH1*, *MSH2*, *MSH3*, and *MLH1*) [17]. These mutations include frameshift mutations in coding regions that can drive oncogenesis by inactivating tumor-suppressor genes or disrupting noncoding regulatory sequences. GCs with high frequency of mutations within microsatellite markers (MSI-high) are characterized by older age, mostly female, distal location, and better survival [18]. The defect in the DNA mismatch repair system of these tumors generates thousands of mutations which may result in a high load of neo-antigens that can be recognized by immune cells [19]. In particular, about 30% of GC was shown to carry a burden of non-synonymous mutations, suggesting that this subgroup of tumors may be particularly responsive to immunotherapy [19]. Of note, MSI-high GC usually shows a prominent lymphocytic infiltrate that is the likely consequence of the high immunogenicity of this subset of GC. The T-cell responses elicited by MSI-high cancers are frequently directed against tumor-specific new carboxy-terminal epitopes originating from short insertion/deletion mutations in coding genes, although frameshift mutations frequently also result in premature arrest of the protein production (stop codon) (Fig. 12.1) [20]. Nevertheless, the same mutator phenotype characterizing the MSI GCs reduces the rates of neo-antigen presentation to the immune system by generating alterations also in genes encoding for MHC class I molecules [21].

Integrated genomic analysis also showed that the two other molecular subtypes of GC, the CIN and GS, are characterized by less evident immune signatures [11, 22], suggesting an inherently reduced responsiveness to immunotherapeutic approaches.

Tumor-Infiltrating Lymphocytes

The composition of the immune microenvironment differs among patients and cancers of the

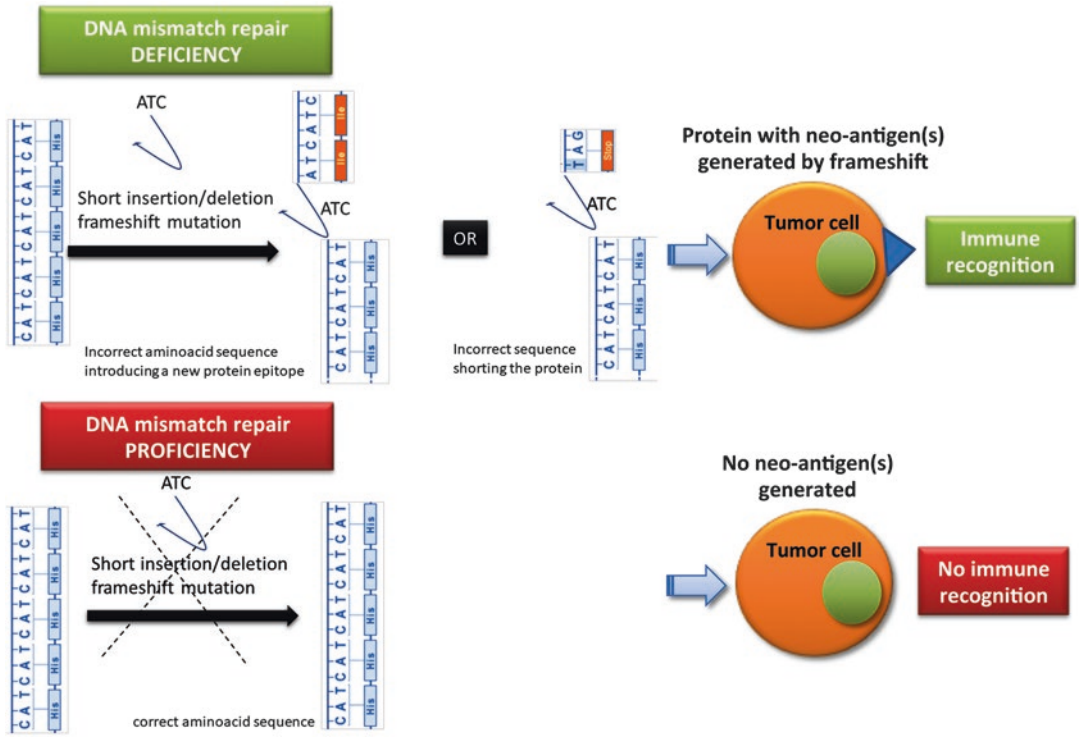

Fig. 12.1 Continuous generation of immunogenic neo-antigens by frameshift mutations not repaired by a deficient DNA mismatch repair machinery, as in the case of MSI-high GC

same type. The nature, number, and spatial distribution of immune cells within the tumor define the host immune background. Several lines of evidence indicate that tumor-infiltrating lymphocytes (TILs) may have an important role in influencing the clinical course of various tumors, also including GC [23]. A higher density of both intratumoral cytotoxic CD8$^+$ TILs and FoxP3$^+$ regulatory T cells (Treg) was associated with good prognosis, and this is particularly true for MSI GC, including those *H. pylori*- or EBV-positive [24, 25]. A recent meta-analysis of 31 observational studies including 4185 GC patients investigated the significance of the prognostic role of specific T-cell subsets, focusing on overall survival and disease-free survival [26]. In particular, the study concluded that the number of CD8$^+$, FoxP3$^+$, CD3$^+$, CD57$^+$, CD20$^+$, CD45RO$^+$, granzyme B$^+$, and T-bet$^+$ infiltrating lymphocytes was significantly associated with improved survival ($p < 0.05$). Notably, the amount of CD3$^+$ TILs in intratumoral compartment was the most significant prognostic marker (pooled HR = 0.52; 95% CI = 0.43–0.63; $P < 0.001$). Infiltrating FoxP3+ Treg cells showed bidirectional prognostic roles, which had positive effect in the intratumoral compartment and negative effect in extra-tumoral compartment [26]. There is an increasing interest in Tregs as one of the major components of the immune-suppressive tumor microenvironment. Treg cells inhibit cytotoxic lymphocytes and/or helper T-cell activity as well as NK cell function, and physiologically they play an important role in maintaining immunological tolerance to self-antigens and in suppressing excessive immune responses that would be deleterious to the host. Tregs have also been identified as the major regulatory component of the adaptive immune response in *H. pylori*-related inflammation, GC, and bacterial persistence [27] as well as in EBV-related GC [25]. A recent study demonstrated that Foxp3$^+$CD4$^+$ICOS$^+$ effector Tregs (eTregs), which have highly suppressive functions, were more abundant in late-stage GCs [28]. These TILs exhibited the ability to produce IL-10 but not IFN-γ, TNF-α, or IL-17 and to inhibit the proliferation of responder CD8$^+$ T cells. The expression of ICOS on Treg cells was found closely related to plasmacytoid dendritic cells expressing ICOS-ligand and TLR9 as well as *H. pylori* infection (Fig. 12.2). These findings suggest that ICOS could be used as a promising target to GC and to eradicate *H. pylori* by an indirect immune therapy [27].

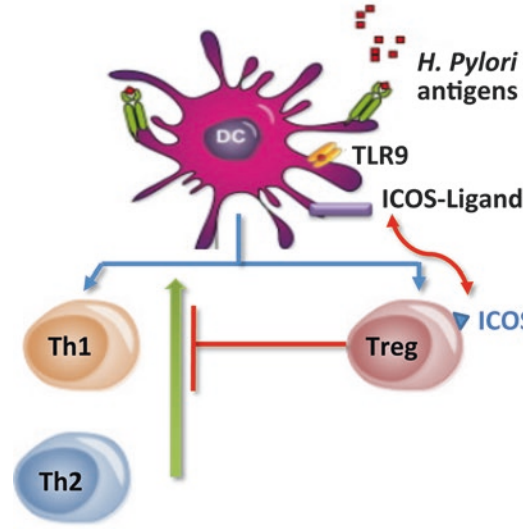

Fig. 12.2 Treg cells expressing ICOS may inhibit the activation of plasmacytoid dendritic cells expressing ICOS-ligand and TLR9 in the setting of *H. pylori* infection. These findings suggest that ICOS could be used as a promising target to eradicate *H. pylori* and treat GC

Tumor infiltration by lymphoid cells is mediated by various mechanisms, including the release of CXCR3 ligands, which behave as chemotactic cytokines whose major function is the recruitment and homing of specific hematopoietic cellular subsets during homeostatic, inflammatory, and neoplastic conditions. CXCR3 is predominantly expressed on activated T lymphocytes, NK cells, inflammatory dendritic cells, macrophages, and B cells and also on tumor and vascular cells. CXCR3 is rapidly induced on naive T cells following activation and remains highly expressed on CD4$^+$ type-1 helper (Th1) T cells, effector CD8+ T cells, and innate-type lymphocytes, such as NK and natural killer T (NKT) cells [29]. CXCR3 expression in GC tissues was significantly higher than in normal adjacent tissues and higher CXCR3 expression correlated with increased dendritic cell and both CD8$^+$ and CD4$^+$ TIL infiltration. By contrast, low levels of

CXCR3 expression were associated with a deeper tumor invasion, III/IV TNM stage, lymph node metastasis, and poorly differentiated tumor cells in GC patients. Notably, univariate and multivariate analyses indicated that CXCR3 expression was an independent prognostic factor for overall survival [30]. Further prospective studies are however required to assess the clinical relevance of CXCR3 overexpression as biomarker of favorable prognosis and therapeutic target in GC.

With regard to other tumor-infiltrating lymphoid populations, it has been reported that in patients with adenocarcinoma of the esophagogastric junction, a high density of tumor-infiltrating B cells, as well as plasma cells, was significantly correlated with better overall survival compared to patients with no infiltrates. B-cell infiltration was as an independent prognostic factor in this study [31].

Overall, the results obtained so far by TILs studies not only support a critical interplay between host immunity and GC but also indicate that the extent and the quality of infiltrating immune cells may have predictive and prognostic significance. As discussed below, ex vivo isolation and expansion of TILs may also constitute a valuable immunotherapeutic approach for GC.

Mechanisms of Immune Evasion in GC

Active cancer immunosurveillance is the ability of host immune system to recognize tumor cells and eliminate them before the development of an overt malignancy [32]. This complex process functions through a mechanism of "immunoediting," which consists of three sequential phases: (1) the *elimination phase*, growing tumors are effectively recognized and eliminated by the concerted action of innate and adaptive immune responses that also recognize remodeling of stroma and changes in the microenvironment. (2) The *equilibrium phase* during which antigen-presenting cells, tumor cells, and $CD8^+$ T cells remain in a state of dynamic balance, and the surviving tumor cells remain quiescent under the pressure of immune cells. In this long phase, the immune system of the host sculpts the immunogenicity of genetically unstable tumor clones, allowing for the selection of resistant tumor cells, thus leading to (3) the *escape phase*, favored by Treg cells and immunosuppressive cytokines including transforming growth factor-β (TGF-β), TNF-α, and IL-10. The immune effector cells in this phase may undergo apoptosis [33].

Tumor cells may activate a variety of mechanisms that help them to interfere with the immune system and avoid detection and killing by immune effector cells. Defects in antigen presentation occur at high frequency in tumors of various origins, including GC, and are a feature of immune evasion that renders cancer cells invisible to cytotoxic T lymphocytes [34]. Selective loss or reduced expression levels of MHC-I or of components of the antigen-processing machinery (APM) are generally associated with disease progression and reduced patient survival [34]. The molecular mechanisms underlying the defect in antigen presentation are diverse and include either irreversible structural alterations or reversible deregulatory processes of APM components [34]. While mutations, deletions, and/or loss of heterozygosity may occur in up to 30% of cases, the expression of APM components in tumors is more frequently deregulated by transcriptional, epigenetic, or posttranscriptional mechanisms [34]. The local microenvironment generated by tumor cells during malignant progression has a major contributory role in functionally impairing antitumor immune responses by promoting the polarization of infiltrating immune cells toward less cytotoxic and pro-inflammatory subsets of T cells (e.g., TH2, TH17, and Treg cells). This process is extremely complex and involves a large number of different cytokines and multiple cellular and stromal cell interactions. In GC microenvironment, the tumor-associated macrophages (TAMs) represent one of the most abundant immune cell populations. These cells can exert antitumor activities or have pro-tumorigenic effects supporting cancer initiation and malignant progression according to differentiation patterns into M1 or M2 subtypes [35]. While M1 TAMs may contribute to tumor control through

the release of pro-inflammatory cytokines (IL-1, IL-6, IL-23, TNF-α), M2 TAMs may drive local immune suppression by producing IL-10 and TGF-β[35]. Indeed, TAM infiltration was shown to functionally inhibit T cells in GC [36, 37] and may represent a biomarker of poor prognosis [38, 39]. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells able to inhibit both innate and adaptive immune responses against tumors [40]. The numbers of MDSCs are increased in the blood of GC patients compared with healthy individuals, and this increase was associated with adverse clinical outcomes [41]. Preclinical evidence indicates that CD40 expression upregulates the chemokine receptor CXCR5 and promotes MDSC migration toward and accumulation within GC tissues [42]. More recently, a multiparametric flow cytometry characterization of $CD45^{+}CD11b^{+}$ $CD14^{+}$ $HLA\text{-}DR^{-}$ MDSCs infiltrating GC disclosed that high numbers of these cells correlated with decreased overall survival and were an independent prognostic factor for overall survival [43].

Another possible immune evasive mechanism is related to the demonstration that GC expresses Fas ligand (FasL) irrespective of tumor stages. This property allows tumor cells to induce a Fas receptor-mediated apoptosis of activated lymphocytes, thus inhibiting antitumor immune responses [44].

Tumor cells may also induce T-cell suppressive signaling pathways to successfully evade immune-mediated elimination. The inhibitory signals to suppress T-cell activity are mediated by a variety of "immune checkpoint" molecules (inhibitory ligands and their cognate receptors), including the CD28/cytotoxic T-lymphocyte antigen 4 (CTLA-4) axis and PD-L1/PD-1 which have emerged as promising druggable targets (Fig. 12.3). Other checkpoint molecules such as TIM3, B7H3, VISTA, LAG3, and TIGIT are currently being evaluated as potential targets for cancer immunotherapy [45, 46]. Pathways involving these regulatory molecules are crucial for maintaining tolerance against self-antigens and to modulate the duration and amplitude of immune responses against nonself or mutated antigens in order to reduce collateral tissue damage [45]. Immune checkpoint molecules play their roles when the immune system recognizes and responds to antigens, mainly provided by infectious agents or cancer cells, and are regulated by ligand/receptor interactions. When these negative regulatory proteins are blocked, the inhibition of immune effector cells is released,

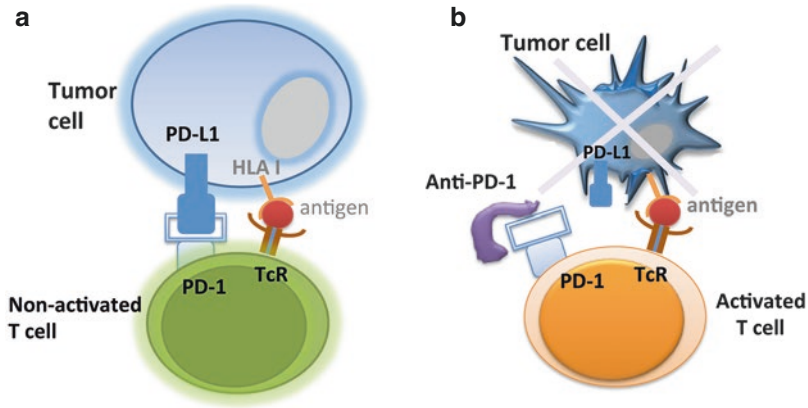

Fig. 12.3 Antibodies/agents against PD-1 receptor on T cells and/or PD-L1 ligand on antigen-presenting cells or tumor cells reactivate pre-existing antitumor T cells that can induce tumor cell killing. Recognition of the human leukocyte antigen (HLA) class I/peptide antigen complex by the T-cell receptor present on T cells is required to induce the tumor cell killing. (**a**) PD-1/PD-L1 interaction is not blocked, and the tumor cell is not killed. (**b**) PD-1 receptor is blocked by an anti-PD-1 antibody, and the T cell is activated and thus able to kill the tumor cell

and these cells regain their ability to become activated and kill tumor cells [47].

About the expression of immune checkpoint molecules in GC, there are variations in methodologies and antibody clones used in immunohistochemistry, clearly pointing to the need to develop and validate standardized approaches [48]. Data collected so far indicate that PD-L1 is expressed in up to 65% of GC tissues, whereas it was undetectable in normal gastric mucosa of healthy individuals [48–50]. A comprehensive immunohistochemical analysis carried out in a series of 127 GCs from Caucasian patients showed that PD-L1 and CTLA-4 were expressed in 44.9% and 86.6% of the cases analyzed, respectively. The load of somatic mutations did not reveal any correlation with the expression of these molecules on tumor cells. Notably, positive tumor cell staining for PD-L1 or CTLA-4 was associated with inferior overall survival. The study also showed that TILs expressed PD-1, PD-L1, and CTLA-4 at significantly higher levels compared to peripheral blood. In addition, PD-1 and PD-L1 were expressed far higher by TILs than CTLA-4 [51]. Despite the efforts carried out so far, the value of PD-L1 in predicting responses of GC patients to anti-PD-1/PD-L1 immunotherapy is controversial. A recent study investigated the expression of PD-L1, PD-L2, and PD-1 as well as $CD8^+$ T-cell density in primary tumors and lymph nodes from patients with stage $T_{1\text{-}4}N_+M_0$ GC. In multivariate analysis, PD-L1 expression, PD-L2 expression, a low density of $CD8^+$ T cells in primary tumors, and PD-1 expression on $CD8^+$ T cells in primary tumors were associated with poor prognosis. In the series analyzed, however, the expression of PD-L1 was heterogeneous in primary tumors and in metastatic lymph nodes, which might explain the inconsistent results in assessing the prognostic value of PD-L1 expression in previous studies [52]. A recent meta-analysis carried out on 15 studies including 3291 GC patients showed that the expression level of PD-L1 in tumor cells significantly correlated with a worse overall survival. In addition, subgroup analysis showed that GC patients with deeper tumor infiltration, positive lymph node metastasis, positive venous invasion, EBV infection, or MSI are more likely to express PD-L1. These findings suggest that GC patients, specifically those with EBV^+ and MSI tumors, may be preferred candidates for PD-1-targeting therapies [53]. Several studies focused on the possible pathogenic and prognostic role of the PD-1/PD-L1 pathway specifically in the subset of EBV-associated GC. Expression of PD-L1 was frequently detected in cancer cells of these GC, usually associated with a stromal infiltration of $PD\text{-}L1^+$ immune cells. Both expression of PD-L1 in cancer cells and $PD\text{-}L1^+$ immune cell infiltration was significantly correlated with diffuse histology. PD-L1 expression in tumor cells correlated with poor outcomes in both overall survival and disease-specific survival. FISH analysis demonstrated gene amplification of PD-L1 in 11% of cases [54, 55]. These results are consistent with the possibility that PD-L1 expression in tumor cells and their microenvironment may contribute to the progression of EBV-associated GC, and gene amplification occurs as clonal evolution during progression.

Expression of PD-L1 by T lymphocytes infiltrating GC may be also of potential prognostic relevance, as shown by a large study carried out on 240 GC patients that found a significantly shorter 5-year overall survival in patients with positive PD-L1 expression on TILs [56]. Expression of PD-1 on NK cells was correlated with a functional hyperresponsiveness of these immune effector cells. PD-1 was found highly expressed on peripheral and tumor-infiltrating NK cells from patients with digestive cancers including esophageal, liver, colorectal, gastric, and biliary cancer. The increased PD-1 expression on NK cells was associated with a shorter survival in esophageal and liver cancers. Functional studies carried out in vitro revealed that blocking PD-1/PD-L1 signaling markedly enhanced cytokine production and degranulation and suppressed apoptosis of NK cells. Intriguingly, treatment with a PD-1 blocking antibody significantly inhibited the growth of xenografts in nude mice, an effect that was completely abrogated by NK depletion [57]. These findings strongly suggested that PD-1 is an inhibitory regulator of NK cells in digestive cancers

and indicated that PD-1 blockade might be an efficient strategy in NK cell-based tumor immunotherapy.

With regard to new immune checkpoint molecules, VISTA appears of particular interest. It encodes for a type I membrane protein and is expressed predominantly on myeloid, granulocytic, and T cells. Although the ligands for VISTA are not yet known, available evidence indicates that VISTA may serve both as a ligand (for antigen-presenting cells) and as a receptor (for T cells) and that VISTA suppresses T-cell activation [58]. In preclinical models, VISTA inhibition increased the number and activation of intratumoral T cells resulting in enhanced antitumor immunity. Of note, VISTA-induced T-cell activation seems to be non-redundantly from the PD-1/PD-L1 pathway [59], suggesting a combined VISTA/PD-1 blockade might be a promising new immunotherapeutic option. Analysis of a large cohort of 464 therapy-naive GC samples and 14 corresponding liver metastases disclosed that VISTA expression in tumor cells was detected in 41 GCs (8.8%) and 2 corresponding liver metastases (14.3%). Moreover, VISTA expression in immune cells was detected in 83.6% of GCs and 42.9% liver metastases. VISTA expression was associated with the Laurén phenotype, tumor localization, Epstein-Barr virus infection, KRAS- and PIK3CA-mutational status, and PD-L1 expression. However, no significant correlation with patient outcome was observed. The concomitant VISTA and PD-L1 expression indicates a dual immune evasion mechanism of GC tumor cells and support the rationale for combination therapies targeting these two immune checkpoint inhibitors in this setting [60].

Tim-3, a member of the TNF family, is a negative regulator of CD4+ helper 1 and CD8+ cytotoxic T cells [61]. The observation that Tim-3 is highly expressed on exhausted or functionally impaired CD8+ T cells suggested a possible correlation between PD-1 and Tim-3 expression and immune evasion in patients with GC [62]. It has been reported that Tim-3 expression defines a subpopulation of PD-1$^+$ exhausted NY-ESO-1-specific CD8$^+$ T cell and that PD-1$^+$Tim-3$^+$ CD8$^+$ T cells represented the largest subset of NY-ESO-1-specific CD8$^+$ T cells in GC patients. Functional analyses disclosed that CD8$^+$PD-1$^+$Tim-3$^+$ T cells were more impaired in IFN-γ, TNF-α, and IL-2 production as compared with PD-1$^+$Tim-3$^-$ or PD-1$^-$Tim-3$^-$ subsets. Concomitant inhibition of Tim-3 and PD-1 during T-cell priming efficiently enhanced proliferation and cytokine production by NY-ESO-1-specific CD8$^+$ T cells, providing thus the rationale for combination immunotherapy targeting these two checkpoint inhibitory molecules [63].

Cancer Vaccines

The therapeutic potential of cancer vaccines is due to their ability to activate and boost antitumor immune responses mainly mediated by T lymphocytes specifically recognizing tumor-associated antigens. The ideal vaccine should be simple and not expensive to produce, easy to administer, safe, and able to induce prolonged protection with a long-lasting memory response. Dendritic cells are professional antigen-presenting cells that play a pivotal role in orchestrating and coordinating antitumor immune responses, being able to activate NK cells, B lymphocytes, and naïve and memory T cells [64]. Tumor antigens processed by DC are loaded in the form of small peptides onto MHC class I molecules for presentation to the cytotoxic CD8$^+$ T cells or to MHC class II molecules for presentation to CD4$^+$ helper T lymphocytes. These functional properties stimulated the development of various strategies aimed at exploiting DC for cancer immunotherapy. Despite these premises, however, the use of DC-based vaccines in the clinical setting is limited by the short life span of these cells in vivo. In GC patients, a higher number of DCs infiltrating the tumor were shown to correlate with lower lymph node metastases and lymphatic invasion and better 5-year survival rates [65–67]. Various tumor-associated antigens were used so far to load DC cells to vaccinate GC patients. Advanced gastrointestinal tumor patients were treated with four injections of

autologous DCs pulsed with melanoma-associated antigen (MAGE) A3 peptides showing the induction of peptide-specific T-cell responses and a minor tumor regression in a proportion of patients [68]. No correlation was however observed between the clinical outcome and the induction of tumor antigen-specific immune responses [68]. An immunogenic HLA-A2 epitope peptide derived from the HER2/neu oncogene was used in a phase 1 clinical trial to pulse autologous DCs and treat a small number of patients with advanced or recurrent GC overexpressing HER2/neu. No severe toxicity was observed, and HER2/neu peptide-specific T-cell recognition could be demonstrated in six of nine patients after immunization. One of the patients underwent a partial clinical response concurrent with a decrease in the blood levels of the carcinoembryonic antigen tumor marker, whereas a stabilization of disease for 3 months was observed in another patient [69]. Peptide-based vaccination strategies for GC have been also investigated in combination with chemotherapy obtaining encouraging results. One study evaluated the effect of adjuvant immunochemotherapy with the use of BCG (bacille Calmette-Guerin) and FAM (5-fluorouracil, Adriamycin, mitomycin C) chemotherapy on the survival of patients with locally advanced resectable GC. In radically resected stage III/IV GC, adjuvant immunochemotherapy significantly prolonged the overall 10-year survival (47.1%) as compared to FAM (30%) or surgery alone (15.2%) [70]. In a multicenter phase II trial, patients with advanced GC or gastroesophageal junction carcinomas were treated with the gastrin-17 diphtheria toxoid (G17DT, Aphton) vaccine targeting gastrin peptide in combination with cisplatin and 5-fluorouracil. Immune responders (61% of the total 94 patients) were identified based on increased levels of anti-gastrin antibody levels on two consecutive tests and showed a significantly longer time to progression and a longer median survival rate compared to non-responders [71]. Recently, the safety and immunogenicity of peptide vaccination with HLA-A*2402-restricted URLC10-A24-177 and VEGFR1-A12-9 1084 epitope peptides were investigated in a phase I clinical trial of patients with advanced GC who were refractory to chemotherapy. No patient had a severe treatment-related adverse event, and specific cytotoxic T-cell responses were detected in 62.5% and 50% of patients for URLC10 and VEGFR1, respectively [72]. To personalize the choice of peptides to be used as vaccines in individual GC patients, pre-vaccination peripheral blood mononuclear cells (PBMCs) were screened for their reactivity in vitro to each of 14 peptides on HLA-A24 or 16 peptides on -A2 allele, and then only the reactive peptides (maximum 4) were administered in vivo. Delayed-type hypersensitivity (DTH) to the vaccinated peptides was observed in four patients, whereas increased cellular and humoral immune responses to the vaccinated peptides were observed in postvaccination PBMCs from four of eight patients and in postvaccination sera of eight of ten patients tested, respectively. Prolonged survival was observed in patients showing cellular and humoral immune responses to the peptides included in the vaccine in the postvaccination samples [73]. The same approach of personalized choice of vaccination peptides was adopted in combination with oral administration of a 5-fluorouracil derivative (TS-1) in a small series of advanced GC. An increase in peptide-specific IgG after the sixth vaccinations was observed in most patients irrespective of the dose of TS-1 used, whereas an increase in peptide-specific interferon-gamma production by T cells was most evident in patients who were administered the highest dose of TS-1. These results indicated that administration of the standard dose (80 mg/m^2/day) of TS-1 in combination with a personalized peptide vaccination does not necessarily hamper immunological responses in GC patients and could maintain or enhance them [74]. Encouraging clinical results were recently obtained by a study in which patients with advanced or recurrent GC were vaccinated with HLA-A24-restricted vascular endothelial growth factor receptor 1 (VEGFR1)-1084 and VEGFR2-169 peptides combined with S-1 and cisplatin chemotherapy. Most patients (82%) showed the induction of VEGFR1-specific cytotoxic T-lymphocyte

responses, 12 patients (55%) showed partial response, and 10 had stable disease after two cycles of the combination therapy.

Notably, patients showing VEGFR-specific T-cell responses had significantly overall survival and time to progression, indicating that cancer vaccination combined with standard chemotherapy warrants further analysis as a promising strategy for the treatment of advanced GC [75]. More recently, a cocktail vaccine including multiple peptides (DEPDC1, FOXM1, KIF20, URLC10, and VEGFR1) combined with S-1 chemotherapy was administered as postoperative adjuvant therapy in a series of pathologically stage III advanced GC patients. The treatment was well tolerated, and the optimal relative dose intensity of S-1 was achieved in combination group, paving the way for further studies aiming at assessing the efficacy of this therapeutic strategy [76].

Adoptive Cell Therapy

The tumor-killing properties of T cells and NK cells provide opportunities to treat cancer. Adoptive cell therapies (ACT) are harnessing this potential by exploiting these effectors, particularly by endowing a functionally diverse repertoire of T cells with genetically modified, tumor-specific recognition receptors [77]. This form of immunotherapy involves the isolation and ex vivo expansion and manipulation of tumor-specific T cells or NK cells, which are then reinfused into cancer patients to combat the disease. This process is applicable to the vast majority of cancer patients who are unable to mount an effective anticancer immunity prior to intervention and therefore at least theoretically will not respond to immune checkpoint inhibitors. Notably, ACT has multiple advantages compared with other forms of cancer immunotherapy that rely on the active in vivo development of sufficient numbers of antitumor immune cells. In vitro activation allows such cells to be released from the inhibitory factors that exist in vivo and that are among the most relevant factors limiting the efficacy of cancer immunotherapy. Moreover, ACT enables the manipulation of the host (e.g., T-cell-depleting chemotherapy) before cell transfer to provide a more favorable microenvironment to efficiently support antitumor immunity. There are several different forms of ACT being used for cancer treatment; most of them have been or are being investigated in the clinical setting for their potential efficacy in GC patients.

Tumor-Infiltrating Lymphocytes (TILs)

Several studies have evaluated the predictive and prognostic relevance of TILs in GC [78]. These cells can infiltrate stroma and tumor cells and are considered the expression of the spontaneous host immune response against the tumor. TILs can recognize cancer antigens that are considered foreign to the body, such as (1) viral proteins, (2) mutated proteins uniquely expressed by tumors (neo-antigens), and (3) cancer germline antigens or fetal proteins that may be aberrantly re-expressed by tumor cells. About GC, MHC class I-restricted T cells specifically recognizing GC antigens were successfully isolated from primary tumors, metastatic lymph nodes, and ascites from a series of GC patients [79]. The different antigen recognition pattern of TILs generated from different sites may have implication for TIL-based adoptive immunotherapy. Although this immunotherapeutic strategy showed promising results in preclinical models, less encouraging findings were observed in the clinical setting except for the treatment of melanoma patients [78]. Indeed, the feasibility of this approach has some important limitations including the limited proportion (about 40%) of biopsies yielding satisfactory T-cell populations and the time (about 6 weeks) required to generate adequate numbers of cells for infusion [78]. Cytotoxic T-cell lines specific for the MAGE tumor antigen and able to recognize and kill GC cells in a HLA-A2-restricted fashion were successfully generated from the spleen of GC patients [80]. These findings suggest that the spleen may have an important role in either clinical tumor vaccination or the treatment of cancer patients by adoptive immunotherapeu-

tic approaches using the tumor-specific peptides [80]. One study reported 13% of complete remissions and 21.7% of partial responses in a series of 23 patients with non-operable advanced GC treated with autologous TILs after in vitro culture with recombinant IL-2 and administered with the same cytokine [81]. The effects of ACT were also investigated in combination with chemotherapy. Patients with GC were treated with expanded activated autologous lymphocytes obtained from peripheral blood after stimulation with anti-CD3 antibody and IL-2. The group receiving this ACT regimen in addition to conventional treatment showed a significantly longer overall survival (27.0 vs. 13.9 months, $p = 0.028$) as compared to patients receiving standard treatment alone [82]. A randomized controlled study investigated the efficacy of T-activated lymphocytes generated from GC patients with IL-2 and administered either intraperitoneally or intravenously in combination with low-dose cisplatin and 5-fluorouracil. The overall survival of patients receiving ACT was significantly better than that of patients treated with chemotherapy alone [83]. A promising strategy to improve the feasibility and efficacy of T-cell based ACT is to use T lymphocytes taken directly from the patient's blood after they have received a cancer vaccine. It has been shown that "priming" rare tumor antigen-specific T cells first, with active immunization, is associated with a more effective expansion of tumor-specific T cells, which can be obtained in greater numbers for therapeutic infusion [78].

Natural Killer (NK) Cells

NK cells have cytotoxic activity against solid tumors and are particularly relevant to prevent the metastatic dissemination of cancer cells. The cytotoxic activity of NK cells is finely regulated through a balance of activating and inhibitory receptors that prevent killing of healthy cells while maintaining effective cytotoxic capacity against neoplastic cells. Therefore, these immune effectors have garnered immense attention as a promising immunotherapeutic agent for treating cancers [84]. Available evidence indicates that individuals with high levels of NK cell cytotoxicity have reduced incidence of cancer and that the infusion of human NK cells into tumor patients may induce significant clinical responses [84]. In a large cohort of GC patients, it has been shown that a high intratumoral infiltration of NK cells, identified by the expression of CD57, correlated with smaller tumors, limited lymph node involvement, and a better 5-year overall survival rate [65]. The positive prognostic significance of NK cell infiltration was confirmed in an independent series of GCs [85]. In keeping with these findings is the observation that a high number of apoptotic NK cell-expressing Fas correlated with cancer progression in GC patients [86]. Among the different approaches attempted so far to exploit NK cells for cancer immunotherapy, the use of in vitro expanded allogeneic NK cells appears particularly promising. Compared to autologous NK cells, allogeneic NK cells are more suitable for quality controls and large-scale production and have the advantage of not being inhibited by self-histocompatibility antigens [84]. NK cells can be successfully expanded from peripheral blood mononuclear cells of healthy donors, in the presence of K562 cells expressing membrane-bound IL-15 and 4-1BB Ligand, and from patients with different solid tumors, including GC [87]. Nevertheless, current approaches of NK cell-based immunotherapy need to be improved to make clinical application more feasible. In this respect, it has been recently shown that PD-1, the well-known immune checkpoint of T cells is highly expressed on peripheral and tumor-infiltrating NK cells from patients with digestive cancers including GC. Blocking PD-1/PD-L1 signaling markedly enhanced cytokine production and degranulation and suppressed apoptosis of NK cells in vitro. Notably, treatment of nude mice carrying tumor xenografts with a PD-1-blocking antibody significantly suppressed tumor growth, an effect that was completely abrogated by NK depletion [57]. These findings strongly suggested that PD-1 is an inhibitory regulator of NK cells in digestive cancers and indicated that PD-1 blockade might be an efficient strategy in NK cell-based tumor immunotherapy.

Cytokine-Induced Killer Cells

Cytokine-induced killer cells (CIK) are a heterogeneous population of immune effector cells that can be easily developed from peripheral blood lymphocytes after stimulation with interferon-γ (IFN-γ), monoclonal antibody against CD3, and interleukin (IL)-2 [88]. These cells exert a potent, non-major histocompatibility complex (MHC)-restricted cytotoxicity mainly through expansion of $CD3^+CD8^+CD56^-$ cells to CD56-positive natural killer (NK) T cells [88]. CIK cell cytotoxicity is mediated by perforin release and dependent on NKG2D recognition and signaling [88] (Fig. 12.4). Notably, CIK cells have also been shown to be effective against multidrug-resistant and FasL-positive malignant cells [85, 89]. Moreover, CIK cells can regulate and increase host cellular immune function in vivo by secretion of cytokines, such as IFN-γ, and several chemokines, including RANTES, MIP-1α, and MIP-1β [85]. Because of their safety and inherently high antitumor activity, CIK cells represent one of the promising cellular immunotherapies. Preclinical studies indicated that CIK cells can exert strong antiproliferative and pro-apoptotic effects in the MGC-803 GC cell line [90] and the MKN74 human GC cell line, mainly releasing IFN-γ and TNFα. In addition, available evidence indicates that ACT in general and therapy with CIK cells in particular benefit from the combination with chemotherapy, which may at least in part overcome the limited GC stroma infiltration of transferred cells [91, 92]. Combination with chemotherapy may also have the advantage of benefitting from the ability of several drugs, including doxorubicin, mitoxantrone, oxaliplatin, and cyclophosphamide, to induce an immunogenic cell death [93]. Indeed, the combination of CIK with oxaliplatin showed superior antitumor effects as compared to monotherapy against drug-resistant GC cells both in vitro and in vivo [94]. Several clinical trials have been carried out so far to investigate the safety and efficacy of CIK therapy in GC patients. A series of 53 patients with stage II–III GC was treated after gastrectomy with autologous CIK cells combined with chemotherapy, and the results were compared with those obtained in 112 GC patients receiving chemotherapy alone. The CIK group showed a significantly improved 5-year OS rate (56.6% vs. 26.8%, $p = 0.014$) and PFS rate (49.1% vs. 24.1%, $p = 0.026$) compared to the control group, and no serious side effect was observed in the CIK group. These results suggested that immunotherapy with CIK cells may serve as an adjuvant treatment to prolong the survival of patients with stage II–III gastric carcinoma [95]. Another study carried out in the adjuvant setting included 151 patients with stage III/IV (M0) GC who had undergone gastrectomy (R0/D2) and who were treated with 6 cycles of adjuvant 5-FU, followed by at least 3 cycles of autologous CIK cells. In the whole series, CIK immunotherapy was associated with a significant improvement in 5-year disease-free survival

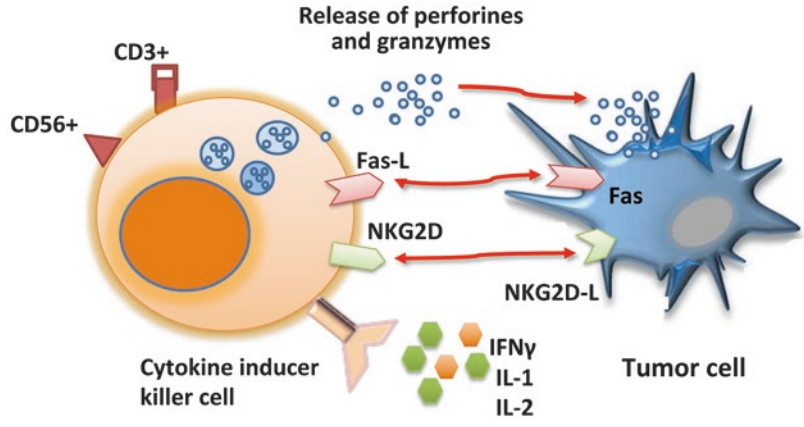

Fig. 12.4 Cytokine-inducer killer (CIK) cells are CD3+ CD56+ cells with a HLA-unrestricted NK cell cytotoxicity. They release perforins and granzymes that kill tumor cells after the recognition of altered cells by a still unknown mechanism. Interaction between NKG2 with its cognate ligand seems to be required for CIK activation and killing

(DFS) rates (28.3% vs. 10.4%; $p = 0.044$), although the difference in overall survival (OS) rates was only of borderline significance. Notably, for patients with intestinal-type GC, the 5-year OS and DFS rates were significantly higher in the CIK group (OS, 46.8% vs. 31.4%; $p = 0.045$; DFS, 42.4% vs. 15.7%; $p = 0.023$). In addition, patients treated with immunotherapy showed increased numbers of $CD3^+$ and $CD4^+$ T cells and increased $CD4^+/CD8^+$ ratio suggesting a reshuffling of T-lymphocyte subset distribution. These findings add further support to the possible clinical benefit provided by CIK cell adjuvant therapy and suggest that intestinal-type GCs could be selected as an important indication for this type of therapy [96]. The efficacy of CIK cell immunotherapy was also investigated in combination with FOLFOX4 in 51 GC patients after gastrectomy. A significant improvement in immune functions was observed in patients treated with CIK and FOLFOX4 compared with the functions of the patients who received FOLFOX4 treatment alone ($P < 0.05$). Notably, the group of patients also receiving CIK treatment showed significantly reduced GC recurrence rates and enhanced survival rates [97]. A meta-analysis considering six relevant clinical trials with case-control studies concluded that CIK cell therapy significantly increased 5-year survival rate compared to conventional chemotherapy among GC patients, thus providing statistical evidence supporting the activation of large-scale clinical trials with CIK cell therapy [98]. The observation that the combination of DCs and CIKs leads to a remarkable increase in cytotoxic activity [99] stimulated the execution of several studies in which this treatment was combined with different chemotherapy regimens. A recent meta-analysis included 1735 GC patients treated with chemotherapy in combination with CIK/DC-CIK within 17 different trials. The analysis showed that the combination therapy significantly increased the OS and DFS rates compared with those of patients treated with chemotherapy alone. The overall response rate ($P = 0.002$), disease control rate ($P = 0.0007$), and quality-of-life improved rate ($P = 0.0008$) were also significantly improved in patients who received the combined treatment. Interestingly, the percentage of lymphocyte subsets ($CD3^+$, $CD4^+$ and $CD3^-CD56^+$, $CD3^+CD56^+$; $P < 0.01$) and the levels of IL-12 and IFN-γ, which reflect immune function, were significantly increased ($P < 0.05$) after the CIK/DC-CIK therapy [101]. Some studies have attempted to identify markers predictive to the response to CIK cell therapy. Of particular interest in this respect is the observation that cell signaling through MHC-I-related chain A (MICA)-natural killer group 2, member D (NKG2D) results in CIK cell activation leading to cytolytic activities against tumor cells [88]. In a cohort of GC patients receiving adjuvant chemotherapy plus CIK or chemotherapy alone, MICA high-expression was found in 36.6% of tumors. Notably, MICA expression significantly correlated with the stage, and there was a borderline association with histological grade ($P = 0.054$). In the adjuvant chemotherapy plus CIK group, GC patients with high MICA expression had longer DFS (46.0 vs. 41.0 months, $p = 0.027$) and OS (48.0 vs. 42.0 months, $p = 0.031$). In multivariate analysis, CIK therapy alone and the interaction of MICA status with CIK therapy were independent prognostic factors for DFS and OS [100]. Although promising, the possible value of MICA status in the clinical decision-making process warrants adequate validation in prospective clinical trials. A particularly attractive perspective for the clinical exploitation of CIK cells is their combination with monoclonal antibodies [101, 102]. Indeed, preclinical evidence has been provided indicating that CIK cells combined with a monoclonal antibody against epidermal growth factor receptor (EGFR) enhanced the antitumor ability of CIK cells both in vitro and in vivo [103].

Chimeric Antigen Receptor T (CAR-T) Cells

To broaden the applicability and enhance the efficacy of adoptive cell therapy, techniques were recently developed to introduce antitumor receptors into normal T cells that could be used for therapy. The specificity of T cells can be redirected by the integration of genes encoding either

conventional alpha-beta TCRs or CARs [104]. The TCR recognition process requires antigen presentation via the major histocompatibility (MHC) complex. However, a significant proportion of tumors downregulate MHC expression to escape immune surveillance [105]. Engineering T lymphocytes with CARs have the advantage to bypass the need for MHC interaction [104]. A CAR T cell can be viewed as the combination of an antibody and a TCR. Its extracellular part is a ligand-binding domain composed of a B-cell receptor-derived single-chain variable fragment, whereas the signaling domain is composed of CD3ζ and one or more intracellular costimulatory domains. Thus, CAR function is independent of MHC presentation or any additional costimulatory signaling [104]. Adoptive transfer of CAR-T cells has so far demonstrated promising antitumor effects in advanced hematologic cancers, such as relapsed or refractory acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma [104]. Nevertheless, in patients with solid tumors, CAR-T cells were able to provide only limited benefit, due to heterogeneous antigen expression, immunosuppressive networks in the tumor microenvironment limiting CAR T-cell function and persistence, and suboptimal trafficking to solid tumors [104]. In a preclinical model of GC, treatment with a humanized chA21 single-chain fragmented antibody (scFv)-based CAR-T targeting HER2 induced a marked regression of HER2-overexpressing tumor and prolonged survival of tumor-bearing mice while spared the progression of HER2 low-expressing tumor [106]. Another CAR-T construct harboring the CD137 and CD3ζ moieties and targeting HER2 exhibited considerably enhanced tumor inhibition ability, promoted long-term survival and homing to targets, compared with those of non-transduced T cells. The sphere-forming ability and in vivo tumorigenicity of patient-derived GC stem-like cells expressing HER2 and the CD44 protein, were also inhibited [107]. To enhance the antitumor activity and in vivo persistence of CAR-T cells, lymphocytes were transduced with a scFv specific for the carcinoembryonic antigen (CEA) in combination with a fusion protein of IL-2. In comparison with free IL-2, the combination of peripheral blood mononuclear cells expressing this CAR and the fusion protein containing IL-2 significantly enhanced the antitumor activity against the human GC cell line MKN-45 cells [108]. This novel combination therapy of CAR and a fusion protein consisting of a functional cytokine and a fully human scFv may be a promising approach for adoptive cancer immunotherapy. Several clinical trials are ongoing to assess the safety and efficacy of CAR-T cells targeting CEA (NCT02349724, NCT02850536, NCT02416466), HER2 (NCT02713984), EpCAM (NCT02725125, NCT03013712), or MUC1 (NCT02617134) in GC patients.

Immune Checkpoint Inhibition

Immune checkpoint inhibitors are certainly the real game changers of modern oncology. The anti-CTLA-4 ipilimumab and the anti-PD-1 antibodies, pembrolizumab and nivolumab, were firstly approved by the US FDA for the treatment of patients with metastatic melanoma in 2011 and 2014, respectively [2, 109]. This class of drugs offers a promising avenue also for GC patients. Data accumulated so far indicate that while anti-CTLA-4 compounds (tremelimumab, ipilimumab) have produced unsatisfactory results, PD-1/PD-L1 inhibitors are showing more promising results.

Anti-CTLA-4 Antibodies

Preclinical and clinical data indicate that drugs targeting the immune checkpoint molecule CTLA-4 function not only by blocking inhibitory signals from reaching effector T cells but also by depleting the Treg cell populations present within the tumor microenvironment [110]. Initial studies in mouse models demonstrated that anti-CTLA-4 antibodies were active in inhibiting tumor growth without inducing serious immune adverse effects [111–113]. The therapeutic efficacy of these drugs was however more limited, if any, in mice bearing tumors with low immunogenicity.

Notably, combination of CTLA-4 blockade with a cellular vaccine transduced with granulocyte-macrophage colony-stimulating factor significantly inhibited the growth of tumors with low immunogenic profile [112]. These findings suggested that CTLA-4 inhibition may be more effective in tumors inherently able to mount a spontaneous antitumor immune response.

Tremelimumab is a fully human IgG2 monoclonal antibody able to inhibit the binding of B7-1 and B7-2 to CTLA-4. A phase II study investigated the efficacy of tremelimumab as second-line therapy in a small cohort of GC patients [114]. The ORR of 5% and the median survival of 4.8 months were quite disappointing, although like those expected with other chemotherapies in GC. Interestingly, patients with a posttreatment carcinoembryonic antigen proliferative response had median survival of 17.1 months compared with 4.7 months for non-responders ($p = 0.004$). These findings support the rationale for combinations of CTLA-4 blockade with vaccines targeting GC antigens. Similar results were observed in a randomized phase II study in which ipilimumab was compared to best supportive care in pre-treated patients with metastatic or locally advanced GC or carcinomas of gastroesophageal junction (GEJ): the survival outcome was similar between the two arms [115].

Pembrolizumab

In the KEYNOTE-012 phase Ib study, single-agent pembrolizumab, a PD-1 inhibitor, was administered to patients with PD-L1$^+$ recurrent or metastatic adenocarcinoma of the stomach/GEJ until progression or intolerable adverse events [116]. Available data indicate that a partial response rate of 22% (8 of 36 evaluable patients) was observed for this group of heavily pre-treated patients, more than 75% of whom had received two or more prior therapies in the metastatic setting. The toxicity observed was manageable, with 13% of patients experiencing grade 3-4 toxicity, and no discontinuation of the therapy due to treatment-related adverse event was recorded.

The KEYNOTE-028 phase Ib study investigated the role of pembrolizumab administered every 2 weeks up to 2 years or until progression in PD-L1$^+$ advanced solid tumors including esophageal/GEJ cancers (adenocarcinoma and squamous cell cancer) [117]. Twenty-three patients were enrolled (the majority of patients were Asian; $n = 12$), with a median follow-up duration of 7 months. Overall response rate (ORR) was 30% (95% CI, 13–53%) and the median duration of response was 15 months (range, 6–26 months). Analysis of a six-gene IFN-γ gene expression signature (*CXCL9*, *CXCL10*, *IDO1*, *IFNG*, *HLA-DRA*, and *STAT1*) analysis suggested that patients with low signature score (non-inflamed) GCs generally had lower response rates and did not show delays in progression. By contrast, delays in progression and increased ORR tended to occur among patients with higher immune gene signature scores [117].

With the aim to improve clinical response rates, PD-1 inhibitors are currently being investigated in combination with chemotherapy in a variety of tumors, including GC. The clinical data of these studies are eagerly awaited after the positive results from a lung cancer trial of pembrolizumab plus chemotherapy [118]. In the KEYNOTE-059 first-line HER2-phase II study, patients with advanced gastric/GEJ adenocarcinoma were treated with pembrolizumab plus 5-FU fluorouracil (or capecitabine in Japan) plus cisplatin every 3 weeks for six cycles, followed by pembrolizumab plus 5-FU/capecitabine maintenance for up to 2 years or until progression [119]. Notably, patients were mainly recruited from the USA (47.9%), only 13.1% from East Asia, and 39.0% from the rest of the world. PDL-1 expression was positive in 57.1% of tumors, and although PD-L1 positivity was associated with a higher ORR of 15.5%, responses were also observed in patients with PD-L1-negative tumors (ORR 6.4%). Indeed, comparable rates of complete responses were seen in the PD-L1 positive (2.0%) and the PD-L1 negative cohort (2.8%). Best responses were seen in patients with MSI-high tumors (four out of seven patients). Overall, treatment with pembrolizumab

resulted in an ORR of 11.6% and the reduction in tumor in 42.4% of patients [119].

KEYNOTE-061 is an ongoing phase III open-label trial evaluating pembrolizumab versus paclitaxel for patients with advanced gastric/GEJ cancer whose tumors have progressed after first-line therapy with a platinum/fluoropyrimidine combination [120]. Treatment will continue until the disease progresses or the drug is no longer tolerated. PFS and OS for patients with PD-L1$^+$ tumors are the primary efficacy endpoints. In the KEYNOTE-062 trial, the efficacy and safety of pembrolizumab alone or in combination with cisplatin plus 5-FU will be compared with chemotherapy alone (cisplatin +5-FU) as first-line therapy for PD-L1$^+$/HER2$^-$ advanced GC or GEJ adenocarcinoma. Primary endpoints are OS and progression-free survival (PFS).

Nivolumab

Like pembrolizumab, nivolumab is a humanized IgG4 monoclonal antibody against PD-1 with activity in multiple tumor types. The phase 1/2 CheckMate 032 trial compared the combination of nivolumab and ipilimumab to nivolumab monotherapy in 160 patients with advanced/metastatic gastric or gastroesophageal cancer [121]. Patients receiving the combination nivolumab and ipilimumab had an ORR of 24%, compared with 12% in patients receiving nivolumab alone. Of interest, ORR in the combination arms seemed to be dependent on dosing, as only 8% patients who received the alternate dosing (nivolumab 3 mg/kg and ipilimumab 1 mg/kg) responded. Responses were observed regardless of PD-L1 expression. As expected from other combination studies, nivolumab + ipilimumab therapy was associated with higher serious toxicity (43%) as compared to nivolumab alone (10%) [121].

ONO-12 (ATTRACTION-2) was a multicenter, double-blind, randomized phase III study of nivolumab for patients with unresectable advanced or recurrent gastric or GEJ cancer refractory to or intolerant of two or more prior chemotherapy regimens (NCT02267343). This was the first randomized, placebo-controlled, phase III trial of immune checkpoint blockade in gastrointestinal cancers. The study showed for the first time that PD-1 inhibition can improve the OSS for patients with heavily pre-treated gastric or gastroesophageal cancer [122]. The observed median OS was 5.32 months (95% CI, 4.63–6.41) with nivolumab vs. 4.14 months (95% CI, 3.42–4.86) with placebo, and the 12-month OS rate was 26.6% (95% CI, 21.1–32.4%) versus 10.9% (HR 0.63; 95% CI, 6.2%–17%; $p < 0.0001$). In addition, median PFS was 1.61 months with nivolumab versus 1.45 months with placebo (HR 0.60; $p < 0.0001$). The ORR rate was 11.2% with nivolumab versus 0% with placebo, and the median duration of response to nivolumab was 9.53 months (95% CI, 6.14–9.82 months) [122]. Patients treated with nivolumab showed a favorable safety profile with treatment-related adverse events (grade 3 or 4) occurring in 34 (10%) of 330 patients, with a frequency similar to those with placebo [122]. It should be considered, however, that the ATTRACTION-2 enrolled only patients from Asian countries, and therefore the results observed might not be applicable to European and North American populations. Recent evidence has suggested the existence of distinct gene signatures associated with inflammation and immunity in GC from Asian and non-Asian patients [123]. A study carried out on a large cohort of more than 1600 GC patients showed that non-Asian GCs were significantly enriched in signatures related to T-cell biology, including CTLA-4 signaling, whereas the immunosuppressive T-regulatory cell marker FOXP3 was significantly enriched in Asian populations.

Avelumab

Avelumab, an anti-PD-L1 IgG1 antibody, is currently being assessed in the JAVELIN trial (NCT01772004) with expansion cohorts for selected tumor types, including gastric/GEJ who had at least one prior therapy or who received avelumab as switch maintenance after chemotherapy [124]. Patients received 10 mg/kg of avelumab every 2 weeks, and preliminary data show

a 9.7% response rate and 6.0 weeks of PFS in the second-line setting. For Japanese patients who had progressed while receiving prior chemotherapy, the reported overall response rate was 15% (3/20 patients), with the proportion of patients' progression-free survival at 12 weeks being 43.3% [125]. The JAVELIN Gastric 300 trial is currently recruiting patients with recurrent, locally advanced, or metastatic gastric/GEJ tumors in an open-label study comparing avelumab to best supportive care in the third-line setting (NCT02625623). Maintenance immunotherapy is being assessed in the JAVELIN Gastric 100 study, which compares single-agent avelumab (10 mg/kg every 2 weeks) to continuation of first-line chemotherapy (NCT02625610) [126]. One recently reported case of metastatic GC who experienced a strong clinical benefit from treatment with avelumab prompted a further characterization of the tumor. The analysis showed no evidence of high mutation burden or mismatch repair defect but disclosed a strong positivity for EBV-encoded RNA. Analysis of The Cancer Genome Atlas GC data (25 EBV^+, 80 MSI, 310 microsatellite-stable, MSS) showed that EBV-positive tumors were MSS and had low-mutation burden but stronger evidence of immune infiltration compared with MSI tumors. Notably, EBV-positive GC had higher expression of immune checkpoint pathway (PD-1, CTLA-4) genes in RNA-seq data and higher lymphocytic infiltration by histology compared with MSS tumors [127]. These findings suggest that EBV-positive low-mutation burden GC are a subset of MSS tumors that may respond to immune checkpoint therapy.

Durvalumab

Durvalumab is a selective, high-affinity, human IgG1κ monoclonal antibody that blocks PD-L1 binding to CD80 and PD-1. Available data indicate that 10 mg/kg of single-agent durvalumab given intravenously every 2 weeks for 12 months showed potential clinical activity in gastroesophageal cancers [128]. Treatment-related adverse events occurred in 33% of patients, with 7% of grade 3 toxicities. A phase IB/II study is currently enrolling patients with GEJ or gastric adenocarcinomas in the second- and third-line metastatic settings for treatment with single-agent durvalumab, single-agent tremelimumab, or combination durvalumab and tremelimumab (anti-CTLA-4) [129].

Combination Strategies Including Immune Checkpoint Blockade

The efficacy of immunotherapies targeting the PD-1/PD-L1 has stimulated the activation of combination studies with other active targeted biologic agents or immune-modulating treatments. The rationale supporting combination immunotherapy is supported by several preclinical data indicating that targeting only one of the complex steps required for the generation of an effective antitumor immune responses is often insufficient. Particularly challenging is the appropriate targeting of the immunosuppressed tumor microenvironment. Preclinical evidence demonstrated that inhibition of the PD-1/PD-L1 axis positively synergizes with antibodies blocking the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) pathway [130]. A recent phase Ia/Ib study investigated the safety and efficacy of the combination of anti-PD-L1 (durvalumab) and anti-VEGFR2 ramucirumab antibodies in patients with refractory GC/GEJ tumors [131]. Preliminary efficacy data showed clinical responses in 3 of 40 (7.5%) patients a 45% disease control rate. Median PFS was 2.10 months for patients treated with ramucirumab 8 mg/kg on days 1 and 2.60 months for patients treated with the same drug given at 10 mg/kg on day 1 only. Ten (25%) patients had grade 3–4 toxicities, most commonly colitis (7.5%) and hypertension (7.5%) [131].

Taking into account the ability of some chemotherapeutic drugs to induce immunogenic cell death, therapeutic approaches combining immunotherapy and chemotherapy are being actively investigated. Based on the promising results obtained with pembrolizumab and considering that PD-L1 is a predictive biomarker for pem-

brolizumab in lung cancer, a phase II study, KEYNOTE-059, was developed to further evaluate pembrolizumab in GC. The multilevel study design included three cohorts: (1) 259 patients with metastatic GC who received pembrolizumab alone, after pre-treatment with two or more lines of chemotherapy; (2) 25 patients with newly diagnosed metastatic GC who received a combination of pembrolizumab and chemotherapy (5-fluorouracil and cisplatin); and (3) 31 patients with newly diagnosed metastatic GC who received pembrolizumab alone. The primary endpoints were safety (all three cohorts) and objective response rate (cohorts one and three) [132]. After a median follow-up of 6 months, an overall objective response rate of 12% was observed in the pre-treated patients (cohort one) who received pembrolizumab alone. Expression of PD-L1 was associated with an increase likelihood to obtain a response (objective response rates of 16% vs. 6%). Many of the responses were durable. Grade 3 to 5 treatment-related adverse events occurred in 18% of patients in cohort one, and 3% had to discontinue treatment as a result [133]. These results are particularly encouraging considering that the expected response rate in these heavily pretreated patients was close to zero. Based on these promising results, the randomized, phase III KEYNOTE-062 study (NCT02494583) was designed to compare the efficacy and safety of pembrolizumab alone or in combination with cisplatin + a fluoropyrimidine with those of cisplatin + a fluoropyrimidine as first-line therapy for PD-L1+/HER2– advanced GC/GEJ adenocarcinoma. The primary study hypotheses are that pembrolizumab in combination with chemotherapy is superior to chemotherapy alone in terms of progression-free survival and overall survival and that pembrolizumab monotherapy is as good as or better than chemotherapy alone in terms of overall survival.

The promising results of these combination studies in advanced disease prompted the activation of multiple trials in patients with earlier-stage disease, including the adjuvant nivolumab phase III trial in resected esophageal and GEJ (CheckMate-577) and a phase I neoadjuvant trial of nivolumab and ipilimumab in stage II–III patients (NCT03044613) [134].

Combination therapies with immune checkpoint inhibitors have also targeted the subset of HER2-overexpressing tumors, which almost invariably become resistant to trastuzumab-containing regimens and progress. Preclinical evidence supports the rationale for combining trastuzumab and inhibitors of the PD-1/PD-L1 axis. In fact, it has been demonstrated that HER2 inhibition can promote T-cell activation and trafficking, enhance IFNγ production by NK cells, and boost antibody-dependent cellular toxicity which may efficiently synergize with inhibition of the PD-1/PD-L1 pathway [135]. A phase Ib/II, open-label, dose-escalation study is investigating the novel anti-HER2 mAb margetuximab in combination with pembrolizumab in patients with advanced HER2-amplified GC who are refractory to standard trastuzumab-based combination chemotherapy (NCT02689284) [136]. A variety of other combinations is being investigated in which, on the backbone of inhibitors of PD-1/PD-L1 axis, other drugs target additional nodes in the cancer immunity cycle [137]. These latter include agents inhibiting other immune checkpoints (TIM3, LAG3), T-cell costimulatory agonist antibodies (GITR, OX40, 4-1BB), enzymatic inhibitors (IDO-1), as well as radiation and other cytotoxic drugs. Additionally, the combination of nivolumab and GS-5745, a matrix metalloproteinase-9 inhibitor, is also being investigated in patients with unresectable or recurrent GC/GEJ adenocarcinoma (NCT02864381). Combination with radiotherapy represents a promising therapeutic opportunity, although still poorly explored in the setting of GC. Single-dose and fractionated radiotherapy can upregulate tumor PD-L1 expression in various preclinical models. Administration of anti-PD-1 antibody concurrently with radiotherapy can overcome the adaptive upregulation of PD-L1 and restore long-term tumor control. Moreover, combination radiotherapy and PD-1/PD-L1 axis blockade demonstrated synergistic antitumor activity and reduce tumor-infiltrating myeloid-derived suppressor cells [138]. An intriguing possibility is that radiotherapy could be used to increase both the necessary antigen

recognition machinery (MHC-I expression) and the magnitude of anti-PD-L1 antibodies binding to tumor cells [138]. Clinical trials involving GC patients are ongoing including studies combining pembrolizumab with palliative radiotherapy in the metastatic setting, as well as with neoadjuvant chemoradiotherapy for GEJ and gastric cardia cancers in earlier-stage resectable disease (NCT02730546) [139].

Concluding Remarks and Future Perspectives

Over the last decade, our understanding of the mechanisms underlying immune modulation has greatly improved, allowing for the development of multiple therapeutic approaches that are revolutionizing the treatment of cancer. Immunotherapy for GC is still in early phases but is rapidly evolving. The challenges moving forward are to put much effort in biologic and immunologic explorations in the GC setting to fine-tune and tailor more precisely the various immunotherapeutic approaches available or emerging. In addition, we must learn how to appropriately integrate immunotherapeutic strategies active against GC with molecularly targeted agents, chemotherapy, and radiotherapy. Rational combinations of different but complementary immune-based approaches should be also carefully investigated with the final goal to offer to each individual the most effective schedule/regimen in relation to the clinico-pathologic, genetic, virologic and immunologic features of his/her own tumor. In this respect, it will be necessary to design large prospective trials to validate reliable predictive factors allowing for the selection of GC patients with the highest chance to benefit from immunotherapy.

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Nanomedicine in Gastric Cancer

13

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Introduction

Gastric cancer (GC), a multifactorial disease, is the fourth most common cancer and the second leading cause of cancer death worldwide. According to geographic areas and sociocultural and economic entities, the distribution of GC varies widely, with the highest rates in East Asia, particularly China, Japan, and South Korea [1]. Many factors are involved in this disease, including infectious, environmental, or host-related factors (age, gender, family history, diet, obesity, tobacco, alcohol, and race). Until now, different therapeutic approaches have already been incorporated to address GC [2]. Current treatment options for GC include a combination of surgery, radiation therapy, and chemotherapy.

Over the past decade, increased knowledge of the tumour microenvironment has facilitated the ability to design new treatments for cancer. Cancer tissue is composed of two compartments: the noncellular (i.e. vascular and interstitial) and cellular compartments surrounded by the normal tissue, which is challenging for local delivery of drugs to tumour cells. Within the noncellular compartment of tumour tissue, regions of fast-dividing cells of the tumour possess a high vascular density, while regions that display tumour necrosis receive little blood supply. Moreover, the tumour cells away from blood vessels have a decreased amount of oxygen. New blood vessels are synthesized by tumours in a process known as angiogenesis.

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*, Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7_13

These vessels are abnormal, with increased numbers of proliferating endothelial cells and vessel tortuosity, deficient pericytes, and abnormalities in the basement membrane with large gaps [3, 4]. Furthermore, the upregulation of vascular endothelial growth factor, bradykinin, prostaglandins, and nitric oxide contributes to the hyperpermeable nature of tumours. Unlike normal tissues, the environment surrounding tumour cells has high interstitial pressure and the absence of a functioning lymphatic network [5]. The combination of deficient lymphatic drainage with increased vascular permeability is responsible for the enhanced permeability and retention (EPR) effect that facilitates the delivery of chemotherapeutic agents to well-vascularized parts of the tumour [6, 7]. However, the drugs may not reach the poorly vascularized regions, thus preventing some cancer cells from receiving cytotoxic treatment. This effect is due to the low microvascular pressure in these regions, which reduces the extravasation of drugs. In addition, the decreased amount of oxygen due to the lack of vasculature results in hypoxic regions of tumours. These regions have a decreased supply of nutrients, such as glucose and essential amino acids. In fact, tumour cells use glycolysis to convert glucose into lactate and obtain the energy that they need to survive and proliferate. In these regions, the formation of lactic acid via anaerobic glycolysis results in an acidic microenvironment. The acidic pH confers resistance against ionized basic drugs. In fact, molecules diffuse passively across the cell membrane most often in the uncharged form. Because the extracellular pH in tumours is low and the intracellular pH of tumour cells is neutral to alkaline, ionized basic drugs that have an acid dissociation constant of 7.5–9.5 are protonated and display decreased cellular uptake [8].

Within the cellular compartment of tumour tissue, there are at least two distinct populations of cells. The first population is made up of a large population of rapidly proliferating cells that form the majority of the tumour mass. The second population is composed of a small rare and quiescent population known as cancer stem cells (CSCs), which are able to regenerate the tumour and retain their genetic programmes for cell migration (i.e. invasion and metastasis) and self-protection differently from non-CSCs that do not have the capacity to self-sustain or metastasize. Most therapeutic treatments target non-CSCs, leaving the CSCs behind, which can then regenerate the tumour, explaining in part why tumours often recur after treatment. Hence, new treatments are being designed to specifically target CSCs, which are now believed to be the critical therapeutic target to prevent local recurrence and metastasis [9]. Inside cancer cells, there are biochemical and metabolic changes that contribute to the cellular mechanisms of drug resistance. Furthermore, the non-specific systemic biodistribution of many chemotherapeutic drugs, resulting in systemic cytotoxicity and lower concentrations of drug delivered directly to the tumour, has limited the full therapeutic benefit of these chemotherapeutic drugs [10].

To overcome these obstacles, new therapies are being designed to deliver chemotherapeutic drugs to the tumour at higher concentrations with a minimal damage to normal tissues using targeting agents conjugated with drugs. However, studies have shown some limitations in their administration to the target sites in vivo, with similar limitations noted for molecular imaging agents [10–12].

New strategies have emerged using nanoparticles (NPs) for drug delivery (therapy), imaging (diagnosis and prognosis), or theranostics for cancer patients [13]. Because of their unique biological properties including their small size, NPs have a high surface area-to-volume ratio, which allows them to bind, absorb, and carry other compounds, such as small molecule drugs, DNA, RNA, proteins, and probes. In addition, their tunable size, shape, and surface characteristics enable them to have high stability, a high carrier capacity, the ability to incorporate both hydrophilic and hydrophobic substances, and compatibility with different administration routes, thereby making them a valuable tool in many aspects of medicine. However, the lack of biodegradation and slow dissolution rates of some NPs raises concern over their safety, especially for long-term administration [14]. NPs can be categorized into those made from biological-like materials (i.e. phospholipids, lipids, dextran, and chitosan), carbon-based materials (i.e. carbon dots), and inorganic NPs (i.e. those based on metals, metal oxides, and metal sulphides), which also include semiconductor NPs (i.e. quantum dots [QDs]).

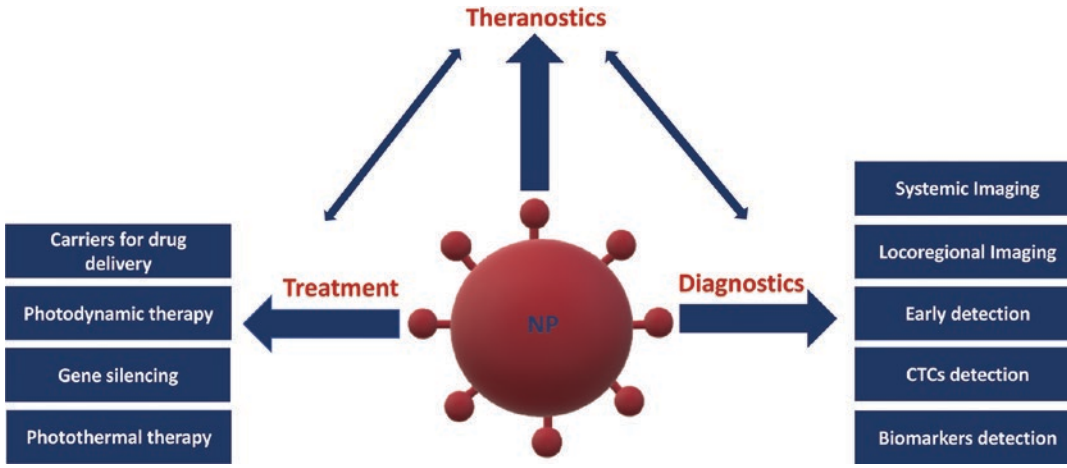

Fig. 13.1 Application of nanoparticles in the treatment, diagnostics, and theranostics of gastric cancer

Depending on the composition, their interaction with cells will be quite different.

In this book chapter, we will discuss the application of NPs in three different fields: treatment, diagnostics, and theranostics of GC (Fig. 13.1). First, we will discuss how NPs are able to function as carriers of chemotherapeutic drugs to increase their therapeutic index and how they can function as therapeutic agents in photodynamic, gene, and thermal therapies. Second, we will discuss the importance of NPs as imaging agents to be applied in systemic and locoregional imaging, early detection and biomarkers, and uncovering circulating tumor cells (CTC). Third, we will describe how NPs could combine diagnosis and therapy as theranostic agents.

Nanoparticles as Carriers for Drug Delivery in GC

The use of nanotechnology in medicine and more specifically drug delivery is helping to revolutionize the treatment of cancer, which includes GC. The NP-drug complex consists of two main components: the NP used as carrier agent and the chemotherapeutic drug [11]. The drug can be adsorbed, dissolved, dispersed, or attached into or onto a nano-matrix. In comparison to conventional formulations, the NP formulation has shown greater bioavailability and a longer sustainable therapeutic time [15]. In addition, the NP formulation overcomes issues, which include low water solubility and severe side effects of drugs. To be effective carriers of chemotherapeutic drugs, NP-drug complexes must fulfil certain criteria:

- The NPs should bind or contain the drug(s).
- The NP-drug complex must remain stable in the serum to accomplish systemic delivery of the drug.
- The NP-drug complex must be delivered only to tumour cells either by receptor-mediated interactions or via the EPR effect.
- The NPs must release the drug once at the tumour site.
- The residual NP must be made of a biological or biologically inert material with a limited lifespan to be safely degraded. If a non-biodegradable material is used, it must be proven to be safe at the doses needed or eliminated from the subject.

After administration into the systemic circulation, NPs loaded with anticancer drugs help to target specifically the tumour either passively or actively and get rid of them without altering or hampering the surrounded non-cancerous tissues [16] (Fig. 13.2). In passive delivery, the EPR effect enables the drug to leave the systemic circulation and enter the extravascular space, where they can accumulate around tumour cells. In active delivery, surface modifications of NPs (i.e. the addition of ligands, such as peptides, small molecules, oligo-

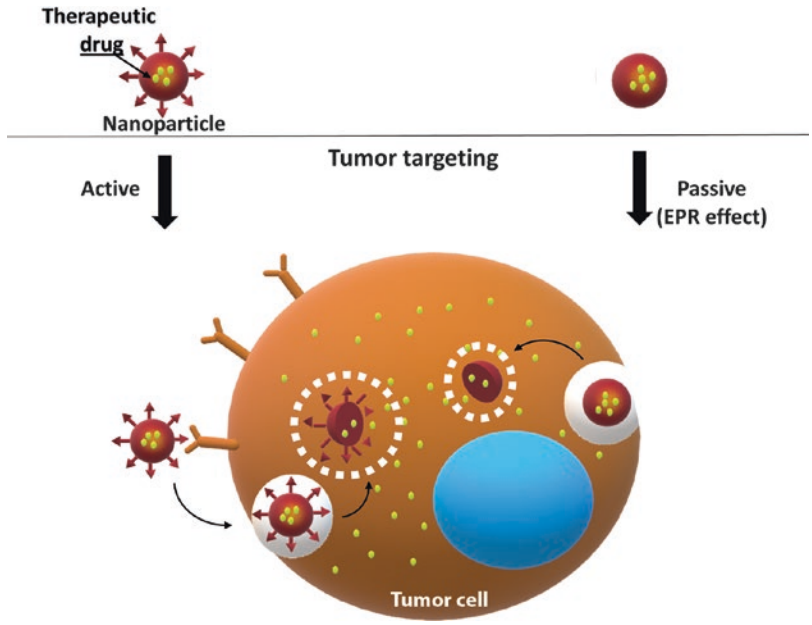

Fig. 13.2 Passive and active targeting approaches of nanoparticles in drug delivery in cancer therapy. Passive tumour targeting (right) is achieved by extravasation of nanoparticles through increased permeability of the tumour vasculature and ineffective lymphatic drainage (EPR effect). Active tumour targeting (left) can be achieved by functionalization of nanoparticles with targeting ligands that promote cell-specific recognition and binding

saccharides, antibodies, and affibodies) allow the NPs to recognize and bind to complementary target molecules on the surface of tumour cells that are overexpressed compared with healthy cells.

Different chemotherapeutic agents have been used to treat GC. According to the National Comprehensive Cancer Network (NCCN) guidelines, 5-fluorouracil (5-FU), cisplatin, and epirubicin continue to be availed as the first-line therapy [17]. Other chemotherapeutic agents have been emerging, including taxanes (docetaxel and paclitaxel), oral fluoropyrimidines (capecitabine and S-1), as well as oxaliplatin and irinotecan [18]. Table 13.1 shows a list of some drugs that are or could be used in GC treatment.

These drugs may work as an individual anticancer drug or in combination [17, 18, 32]. Despite their anticancer properties, these agents still show several side effects in humans [34]. In addition, their delivery method is sometimes difficult. Therefore, the use of NPs has become practical as they could reduce side effects and improve the efficacy of the treatment.

A wide range of studies have already been performed by different researchers to demonstrate the effectiveness of NPs as carriers for drug delivery to treat GC. Table 13.2 shows a list of some NPs that are used for drug delivery in GC treatment.

Camptothecin (CPT) is an effective anticancer drug that is used in multiple types of cancer and has shown remarkable anticancer activity in animal tumour models [35]. It is a DNA topoisomerase 1 inhibitor [36, 37]. However, its clinical utility is limited by its poor solubility and high systemic toxicity. The analogues of CPT, e.g. irinotecan (IRN) and topotecan (TPT), overcome some of its limitations, but they still have suboptimal toxicity profiles and pharmacokinetics. For this purpose, a NP combination, CRLX101, containing a cyclodextrin-based polymer and CPT, was evaluated [38]. The results showed that in a xenograft mouse model utilizing the GC cell line BGC823, CRLX101 is safe, effective, and more bioavailable than the parental drug. Another anticancer drug, docetaxel (DOC), has been reported to exhibit radio-enhancement efficacy in different cancers, including GC [39–41]. However, its applicability remains to be improved because of its non-specific distribution that demonstrates several side effects, such as myelosuppression, neurotoxicity, and musculoskeletal toxicity [42]. To solve this problem, DOC-loaded poly(ethylene glycol) (PEG)-poly(ε-caprolactone) (PCL) NPs were targeted with gelatinase to a radioresistant cell population. Comparing the radiosensitization effect on GC between DOC-NPs and DOC

Table 13.1 Drugs for gastric cancer treatment

Class	Name	Target	Action	Administration route	References
Glutamic acid derivatives	Raltitrexed	Thymidylate synthase	Inhibitor	IV	[19, 20]
	Pemetrexed	Thymidylate synthase	Inhibitor	IV	[19, 21]
		Bifunctional purine biosynthesis protein PURH	Inhibitor		
		Dihydrofolate reductase	Inhibitor		
		Trifunctional purine biosynthetic protein adenosine-3	Inhibitor		
	Leucovorin	Folic acid targets	Folic acid analogue	IV	[22]
	Methotrexate	Dihydrofolate reductase	Inhibitor	IV	[23]
Platinum derivatives	Cisplatin	DNA	Cross-linking/alkylation	IV	[23, 24]
	Carboplatin	DNA	Cross-linking/alkylation	IV	[24]
	Oxaliplatin	DNA	Cross-linking/alkylation	IV	[23, 24]
Anthracyclines	Doxorubicin	DNA	Intercalation	IV	[25]
		DNA topoisomerase 2-alpha	Inhibitor		
	Epirubicin	Chromodomain-helicase-DNA-binding protein 1	Antagonist	Oral	[25, 26]
		DNA topoisomerase 2-alpha	Inhibitor		
		DNA	Intercalation		
Mitomycins	Mitomycin	DNA	Antagonist Cross-linking/alkylation	IV	[27]
Camptothecins	Irinotecan	DNA topoisomerase I	Inhibitor	IV	[28]
	Topotecan	DNA	Intercalation	IV	[28]
Fluoropyrimidines	5-FU	Thymidylate synthase	Inhibitor	IP	[23, 29]
		DNA/RNA	Incorporation into and destabilization		
	Capecitabine	Thymidylate synthase	Inhibitor	Oral	[29]
		DNA/RNA	Incorporation into and destabilization		
	S-1	Thymidylate synthase	Inhibitor	Oral	[29]
		DNA/RNA	Incorporation into and destabilization		
	Doxifluridine	Thymidylate synthase	Inhibitor	Oral	[30]
		DNA/RNA	Incorporation into and destabilization		
	UFT	Thymidylate synthase	Inhibitor	Oral	[31]
		DNA/RNA	Incorporation into and destabilization		

(continued)

Table 13.1 (continued)

Class	Name	Target	Action	Administration route	References
Taxanes derivatives	Paclitaxel	Apoptosis regulator Bcl-2	Inhibitor	IV	[32]
		Tubulin beta-1 chain	Inhibitor		
		Nuclear receptor subfamily 1 group I member 2	Inducer		
	Docetaxel	Nuclear receptor subfamily 1 group I member 2	Binder	IV	[32]
Matrix Metalloproteinase Inhibitors	Marimastat	Matrix metalloproteinase	Inhibitor	Oral	[19]
Tyrosine kinase inhibitors	Lapatinib	Epidermal growth factor receptor	Antagonist	Oral	[33]
		Receptor tyrosine-protein kinase erbB-2	Antagonist		
	Foretinib	Vascular endothelial growth factor receptor 2	Antagonist	Oral	[33]
		Hepatocyte growth factor	Antagonist		
	Sunitinib	Platelet-derived growth factor receptors	Inhibitor	Oral	[33]
		Vascular endothelial growth factor receptors	Inhibitor		
		stem cell factor receptor	Inhibitor		
		Fms-like tyrosine kinase-3	Inhibitor		
		Colony-stimulating factor receptor type 1	Inhibitor		
		Glial cell line-derived neurotrophic factor receptor	Inhibitor		
Monoclonal antibodies	Ramucirumab	Vascular endothelial growth factor receptor 2	Antagonist	IV	[33]
	Pertuzumab	Receptor tyrosine-protein kinase erbB-2	Antibody	IV	[33]
	Trastuzumab	Receptor tyrosine-protein kinase erbB-2	Antibody	IV	[33]

I.V. Intravenous, *I.P.* Intraperitoneal

Table 13.2 Nanoparticles for drug delivery in GC treatment

Type of nanoparticle	Use	Anticancer strategy	Stage of development	References
Cyclodextrin-based polymeric NPs	Carrier	CPT	In vivo/in vitro	[38]
Gelatinase-stimuli PEG-Pep-PCL NPs	Carrier	DOC	In vivo/in vitro	[43]
γ-PGA-based NPs	Carrier	Co-loading of CET and DOC	In vivo/in vitro	[48]
HA-modified layer-by-layer NPs	Carrier	Co-loading of IRN and 5-FU	In vivo/in vitro	[49]
Amphiphilic mPEG-PCL block copolymer NPs	Carrier	UA	In vitro	[53]
Liposomes	Carrier	Poly(I:C)	In vivo/in vitro	[59]
Lipid-coated nanodiamond	Carrier	Sorafenib	In vivo	[65]
Copolymer PMMA-AA encapsulated ZnO NPs	Carrier	Cur	In vitro	[69]
CO NPs	Anticancer agent	Upregulation of DHX15 protein	In vivo/in vitro	[70]
CH NPs	Anticancer agent	Proliferation inhibition	In vitro	[72]
CH-HA-coated SWNTs	Carrier	SAL	In vitro	[84]
Beta-casein NPs	Carrier	PTX	In vitro	[85]
PLGA NPs coated with hCTLs membranes	Biomimetic delivery system	PTX	In vivo/ex vivo	[93]

in vitro and in vivo revealed a significant increase in the radiosensitivity of DOC-NPs in all three gelatinase-overexpressing GC cells (BGC823, SGC7901, and MKN45 cell lines) compared with normal mucosa cells. In addition, the radiosensitization efficacy of DOC-NPs was more prominent than DOC by intravenous injection in a xenograft. Thus, gelatinase-mediated nanoscale delivery system could serve as a potential strategy to increase the radiosensitization and the specificity of DOC as well as to reduce its side effects [43].

The epidermal growth factor receptor (EGFR), which is upregulated in GC patients, is an oncogene that has reported to be an indicator of a poor prognostic outcome and, in turn, an important therapeutic target [44]. Cetuximab (CET) is a chimeric IgG monoclonal antibody (MAb) directed against EGFR. It was approved by the US Food and Drug Administration (FDA) for the use in colorectal carcinoma, and it has also shown clinical benefit towards advanced/metastatic gastric adenocarcinoma in combination with other chemotherapeutic agents as a first-line treatment [45–47]. To enhance the therapeutic efficiency of conventional chemotherapeutics, a study reported the development of CET-conjugated DOC-loaded poly(γ-glutamic acid) (γ-PGA) NPs (CET-DOCT- γ-PGA-NPs) [48]. Compared with non-targeted and free drug formulations, CET-DOC- γ-PGA-NPs showed EGFR-specific cellular internalization and significant cancer cell death in vitro induced by active targeting. In addition, this system showed improved systemic circulation and enhanced tumour accumulation by passive targeting due to the EPR effect of the tumour environment and EGFR-mediated cellular internalization, enhancing the drug availability at the tumour site and resulting in tumour growth inhibition in vivo in the MKN-28 GC xenograft model. Thus, the combination of the targeting agent CET and the therapeutic agent DOC, with the γ-PGA nano-matrix, was found to be an effective targeted nanoformulation for EGFR-overexpressing GCs.

To target GC, hyaluronic acid (HA)-modified layer-by-layer NPs were used for the co-loading of IRN and 5-FU to improve anticancer treatment efficacy and reduce side effects [49]. A polymer-chitosan (CH)-HA hybrid formulation (HA-CH-

IRN/5-FU NPs) consisting of poly(lactic-co-glycolic acid) (PLGA) and IRN as the core, CH and 5-FU as a shell, and HA as the outermost layer was prepared. Human gastric carcinoma cells (MGC803 cells) and cancer-bearing mice were used to test the in vitro cytotoxicity and in vivo antitumour efficiency of HA-CH-IRN/5-FU NPs. The results demonstrated that this targeted drug delivery system has an impressive antitumour activity in vitro and in vivo.

Cyclooxygenase-2 (COX-2), an inducible isoform of cyclooxygenase, is regulated by cytokines and growth factors, such as IL1β, IL6, or TNFα. This isoform is overexpressed during inflammation, constitutively expressed in GC, and associated with tumour progression [50]. It has been shown that ursolic acid (UA) can induce apoptosis of cancer cells by inhibiting the expression of COX-2 [51, 52]. UA is relatively nontoxic, but its clinical application is limited due to several problems, including poor water solubility leading to low bioavailability and poor pharmacokinetics, subsequently restricting its effectiveness and its non-specific distribution throughout the body when administered intravenously. To overcome these limitations, UA-loaded NPs (UA-NPs) were prepared using amphiphilic methoxypolyPEG-PCL (mPEG-PCL) block copolymers as drug carriers. This nano-drug delivery system effectively transported UA into SGC7901 cells and increased cell apoptosis to improve the anticancer efficiency of UA [53].

Several immunoagents have been developed as anticancer drugs. Among them, poly(inosinic-cytidylic) acid or poly(I:C), a synthetic analogue of double-stranded RNA (dsRNA), has been found to trigger apoptosis in a variety of cancers [54–58]. However, there have been few studies on GC. A study has shown that intracellular delivery of poly(I:C) by liposomes has a pro-apoptotic effect on human gastric adenocarcinoma cells in vitro and significantly inhibits xenograft tumour growth of human gastric adenocarcinoma in nude mice [59].

Approximately 80–90% of patients diagnosed with GC present metastasis [60–62]; in turn, it is a great challenge to improve the survival rate. Sorafenib is an oral molecular targeting agent with anti-proliferative and antiangiogenic activities. It is a potential agent for the treatment of metastatic GC [63]. However, it is almost insoluble in water, and its oral bioavailability is extremely low, which greatly restricts its therapeutic efficacy towards cancer metastasis. Nanodiamond, a member of the carbon NP family, is characterized by a large surface area, high adsorption capacity, and good biocompatibility, which makes it attractive for drug delivery and cellular imaging [64]. A study has shown that lipid-coated nanodiamonds loaded with sorafenib can increase the oral bioavailability of the drug and its efficacy in the suppression of GC metastasis in tumour xenograft models [65].

Curcumin (Cur) is a well-known phytochemical that demonstrates antitumour activity in many human cancers including GC. It can induce cell apoptosis, especially in malignant cells, by causing DNA damage. Cur is safe even at high doses, but its utility is limited due to poor aqueous solubility and oral bioavailability and multidrug resistance [66, 67]. Thus, creating a drug delivery system with enhanced drug solubility leading to improved bioavailability and efficacy is an exigency. ZnO NPs with a tunable size and shape have many advantages to construct such drug delivery systems due to their reduced toxicity and stability towards the environment [68]. ZnO itself is nontoxic, but after decomposition, Zn^{2+} ions are cytotoxic. A study successfully developed a copolymer, PMMA-AA encapsulated ZnO NPs, for the loading of Cur and tested it in AGS GC cell lines in vitro [69]. Compared with free Cur, Cur/PMMA-AA/ZnO NPs enhanced cellular uptake and reduced the cytotoxicity of Cur; rapid release of the payload was observed at a low pH and with high bioavailability. Therefore, the Cur/PMMA-AA/ZnO NPs constitute an alternative method to enhance the anticancer activity and delivery of hydrophobic anticancer drugs with biocompatible and pH-sensitive nano-vehicles.

Another well-known anticancer agent, cerium oxide NP (CO NP), has been tested in human GC cell lines (MKN28 and BGC823) and GC xenografts. In fact, research data have shown diverse abilities of CO NPs, including antioxidant capa-

bilities and cancer cell sensitization to radiation therapy and chemotherapy. The results suggest that CO NPs have an inhibitory effect on cell migration and proliferation both in vitro and in vivo by increasing the expression of putative ATP-dependent RNA helicase DEAH (Asp-Glu-Ala-His) box helicase 15 (DHX15). This protein can activate the p38 MAPK signal pathway and in turn inhibit proliferation and metastasis. The inhibitory effect of CONPs is dose-independent, whereas their effect on proliferation is dose-dependent and only a relatively high concentration of CONPs suppresses proliferation [70].

CH NPs are widely studied NPs in anticancer treatment because of many advantages, including particle size, zeta potential, morphology, safety, bioavailability, and biocompatibility [71]. A study aiming to identify the effects of these NPs on the proliferation of the human GC cell line MGC803 in vitro has shown that after treatment with CH NPs, these NPs are cytotoxic and effectively inhibit cell proliferation through multiple mechanisms [72].

GC stem cells (CSCs) play a pivotal role in the initiation, development, relapse, and metastasis of GC because they are resistant to standard chemotherapy, and the residual CSCs are able to proliferate indefinitely [73]. Therefore, eradication of this cell population has important clinical implications in cancer therapy. Several studies have identified CD44 as a cell surface marker of gastric CSCs [74–77]. HA has been identified as a potent targeting ligand of tumours possessing CD44-overexpressing cells [78]. Several findings have strongly suggested that salinomycin (SAL), an anticancer drug, might represent a class of agent for targeting CSCs [79–82]. However, its poor aqueous solubility [83] has limited its application. Therefore, to overcome the poor solubility as well as improve the biodistribution to yield superior drug encapsulation and accumulation in tumours, a research group assembled a gastric CSC targeting drug delivery system [84]. This system is based on CH-coated single-wall carbon nanotubes (SWNTs) loaded with SAL functionalized with HA (SAL-SWNT-CH-HA), which helps to minimize the movement and intrusion of GC stem cells as well as their eradication.

Paclitaxel (PTX) is a widely used chemotherapeutic agent but with undesirable side effects during injection since it needs to be delivered intravenously. Different approaches to administer PTX orally have been attempted by using organic and synthetic delivery systems, but they remain unsuccessful. A study evaluated a potential drug delivery system composed of the hydrophobic anticancer drug PTX entrapped within beta-casein (b-CN) NPs, which can be degraded by the stomach enzyme pepsin [85]. After encapsulation and simulated digestion with pepsin, PTX maintained its cytotoxic activity towards human N-87 GC cells, whereas, without prior simulated digestion with pepsin, the b-CN-PTX NPs were non-cytotoxic. These data suggest that b-CN may protect upper gastrointestinal regions from PTX and efficiently release it in the stomach without compromising drug cytotoxicity. It has been found that this system shows promise to be useful for target-activated oral delivery of hydrophobic chemotherapeutics in the treatment of gastric carcinoma. The integration of synthetic (inorganic or organic) NPs in GC treatment has shown many advantages, including an increased chemotherapeutic drug concentration at tumour sites, decreased systemic exposure, and an EPR effect because of their passive accumulation ability and targeting ligand incorporation on their surface [86–88]. However, their short circulation time, the complexity of producing such actively targeted carriers, the difficulty of heterogeneous and varied tumour vascularization and tumour permeability with different tumour types and stages, and their unknown toxicity have limited their clinical utility. Therefore, combining synthetic NPs with natural biomaterials to create biomimetic delivery systems is becoming more attractive because of their ability to mimic many features of their source cells as a new engineering strategy [89–92].

To enhance PTX targeting in GC treatment, a study aimed to produce a biomimetic system based on human cytotoxic T lymphocyte (hCTL) membranes because of the long blood circulation time and ability to recruit and localize at tumour sites of this cell type [93]. In this platform, local low dose irradiation (LDI), which induces the expression of

adhesion molecules and chemoattractants [94, 95], was used to guide PTX-loaded PLGA NPs coated with cellular membranes isolated from hCTLs. After systemic administration, this new system reduced NP phagocytosis by macrophages to 23.99% and inhibited the growth of human GC by 56.68% in Balb/c nude mice. The application of LDI at the tumour site significantly increased the tumour growth inhibition rate to 88.50%, and two mice achieved complete remission. Combining ex vivo experiments with in vivo experiments, this new drug delivery platform favoured both the long circulation time and the tumour site accumulation ability of hCTLs, while local LDI significantly enhanced tumour localization. This LDI-guided biomimetic drug delivery platform provides a promising system for cancer immunotherapy, photothermal therapy, and diagnosis in the near future.

Nanoparticles for RNAi Delivery

Gene silencing is the regulation of gene expression to prevent the expression of a certain gene. For the silencing of gene expression, antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) are the two most widely used strategies [96]. siRNA holds great promise in cancer treatment, as numerous studies have shown that the growth and proliferation of cancer cells can be greatly inhibited by using this approach in vitro and in vivo [97–99]. In addition, different siRNAs are able to silence not only one but several oncogenes with high efficacy and specificity, allowing the simultaneous targeting of multiple pathways. Moreover, siRNA-based therapeutics have demonstrated great potential in sensitizing cancer cells to chemotherapy by silencing genes that play a role in drug resistance during chemotherapy [100, 101]. However, several limitations reduce the therapeutic efficacy of siRNA, including delivery problems, side effects due to off-target actions, and others [102]. In addition, unmodified siRNA molecules are highly unstable when delivered into the systemic circulation and are unable to enter cells due to their size and the high polyanionic charge of the phosphate backbone. Therefore, delivery systems such as NPs are currently being explored as an alternative way to safely transport siRNA (Fig. 13.3).

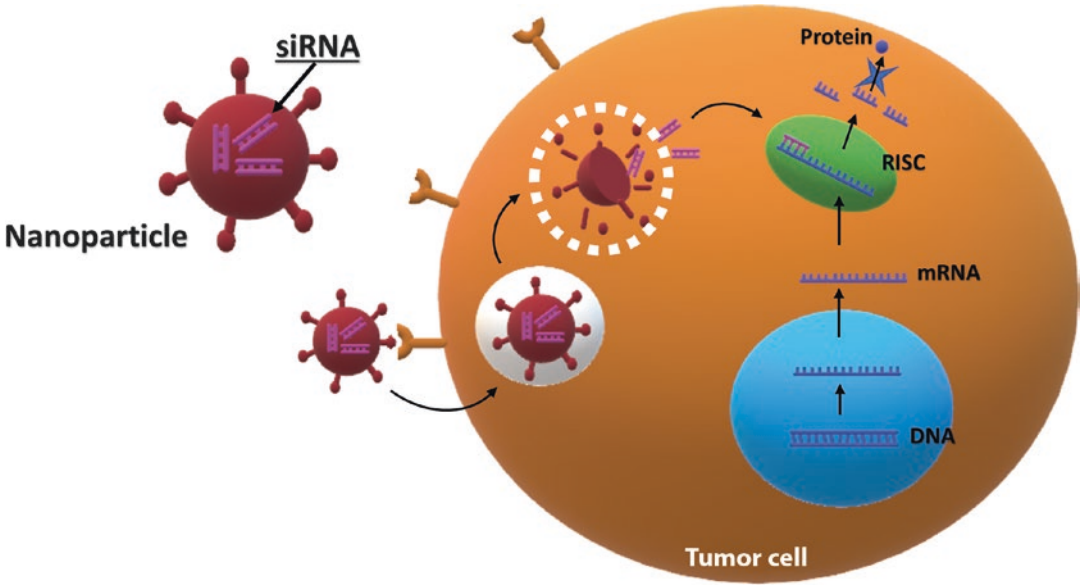

Fig. 13.3 Nanoparticles in gene therapy. Nanoparticles can deliver small interfering RNAs (siRNAs) into tumour cells, where they can interact with the translation of a targeted messenger RNA (mRNA) molecule. Delivered siRNAs are processed by specific RNA-induced silencing complex (RISC), the double-stranded RNA is unwound, and one strand is degraded, whereas the other strand binds to the targeted mRNA by complementarity, leading to its degradation by endonuclease activity and thus silencing gene expression

Table 13.3 Nanoparticles used as delivery systems for gene silencing in GC treatment

Type of nanoparticle	Use	Anticancer strategy	Stage of development	References
AMO-21-HER-PEG-PCL NPs	Carrier	MiR-21	In vivo/in vitro	[107]
Folate-conjugated 3WJ-BRCAA1 siRNA-pRNA NP system	Carrier	BRCAA1	In vivo/in vitro	[115]
PEG-modified PEI NPs	Carrier	CD44v6	In vitro	[116]
Calcium phosphate NPs	Carrier	Suicide genes	Ex vivo	[117]

NPs have a large surface area-to-volume ratio and thereby can carry and protect siRNA from degradation, specifically targeting and delivering siRNA to cancer cells after functionalization with tissue-specific ligands. In addition, NPs are efficiently taken up into cells through membrane fusion or receptor-mediated endocytosis. Table 13.3 shows some studies that utilized nanoparticles as delivery systems for gene silencing in GC treatment.

Despite the advantages of combination chemotherapy, approximately 7–34% of GC are characterized by a poor prognosis associated with amplification of the human epidermal growth factor receptor 2 gene (HER2) [103–106]. MiR-21 is a microRNA that is frequently overexpressed in GC, decreasing the sensitivity of GC cells to trastuzumab, which is a humanized MAb targeting HER2. A study used PEG-PCL NPs coated with trastuzumab to target GC cells, which overexpressed HER2 receptor using anti-miRNA-21 antisense oligonucleotides (AMO-21) [107]. The antibody conjugates significantly enhanced cellular uptake of NPs. The HER-PEG-PCL NPs effectively suppressed miRNA expression in GC cells, resulting in enhancing sensitivity of HER2-expressing GC cells to trastuzumab. The biological and clinical potential of targeted AMO-21 delivery using modified trastuzumab for GC treatment have been shown by comparing the antitumour effects of AMO-21-HER-PEG-PCL NPs with trastuzumab in xenograft GC mice.

As mentioned before, it remains very difficult to effectively cure GC patients because most of them present advanced stages of the disease. Advanced and metastatic cases do not respond to chemo- or radiation therapies [108, 109]. Resistance to chemotherapy-induced apoptosis is a major cause for the failure of conventional therapies [110, 111]. The current prognosis of GC is very poor, with 5-year survivals of less than 24% [112]. Therefore, how to recognize, track or kill early GC cells is a great challenge for patients with early GCI. Breast cancer-associated antigen 1 (BRCAA1) is overexpressed in GC with no expression in normal control gastric mucous tissues [113, 114], strongly suggesting that BRCAA1 antigen may be selected as a potential target for early GC. A research group has successfully designed a folate-conjugated three-way junction (3WJ)-BRCAA1 siRNA-packaging RNA (pRNA) NP system [115]. RNA NPs can enter the cytoplasm specifically via folic acid (FA) receptor-mediated endocytosis and inhibit BRCAA1 expression in GC to induce GC MGC803 cells and reduce the burden of tumour xenografts in vivo. Therefore, RNA nanotechnology provides a promising strategy that can overcome conventional cancer therapeutic limitations due to the specific delivery of therapeutics to stomach cancer without damaging normal cells, reduce toxicity and side effects, and improve the therapeutic effect.

siRNA is an efficient tool to suppress the activity of CD44v6, a protein involved in the progression of GC. However, its potential for clinical therapy has been limited due to its instability and low transfection efficiency. To bypass these limitations, a research group studied and synthesized poly(ethylene glycol)-poly(ethyleneimine) (PEG-PEI) NPs, a non-viral carrier of siRNA targeting CD44v6 in SGC7901 human gastric carcinoma cells. This non-viral carrier may be a promising system for altering gene expression in the treatment of GC, exhibiting many advantages, such as a relatively high gene transfection efficiency and low cytotoxicity [116].

In another gene therapy in vivo approach, calcium phosphate NPs were combined with suicide

genes, e.g. bCD (bacterial cytosine deaminase), to test the efficacy of these NPs against GC [117]. The expression of suicide genes delivered by these NPs in specific GC tissues inhibits gastric carcinoma growth.

Nanoparticles as Therapeutic Agents in GC treatment

Photodynamic Therapy

Photodynamic therapy (PDT) has recently emerged as an attractive approach for the treatment of several types of cancers, particularly GC [118] (Fig. 13.4).

PDT uses drugs called photosensitizers (PSs) and a particular type of light. When PSs are exposed to a specific wavelength of light, they generate cytotoxic oxygen-based molecular species that cause damage to plasma membranes and subcellular organelles, resulting in cell death either by apoptosis, necrosis, or autophagy. PSs are able to transfer the absorbed energy from light to either oxygen molecules to produce singlet oxygen or to surrounding molecules to form free radicals. The effectiveness of PDT depends on the capacity of PSs to generate singlet oxygen and their ability to be selectively delivered at therapeutic concentrations to the target tumour tissue [119]. However, there are several difficulties associated with the use of PSs in PDT, such as low accumulation in specific target cells, environmental degradation, and a short lifespan of singlet oxygen species [119–121]. To overcome these problems, various NP-based systems have been investigated. NPs used in PDT can be functionally divided into passive or active. Passive PDT NPs are carriers of PSs and can be made from either biodegradable material or non-polymer-based materials, such as ceramic and metallic NPs. Active PDT NPs can produce reactive species without the presence of PSs.

Several studies have shown that NPs could be potential carriers of PSs to improve PDT in the treatment of GC. Table 13.4 provides a list of some NPs involved in PDT for the treatment of GC.

For example, a study has focused on the development of a biodegradable NP system based on polyethylene glycol-modified gelatine (PEG-GEL) and poly(lactic acid) (PLA) biopolymers as a carrier for a potent PDT agent, cyclohexane-1,2-diamino hypocrellin B (CHA2HB), to improve its photodynamic efficacy [122]. In vitro experiments indicated that CHA2HB-loaded PEG-GEL-PLA NPs were efficiently taken up by AGS human gastric carcinoma cells and induced both apoptotic and necrotic cell death as a result of photoirradiation, suggesting that PEG-GEL/

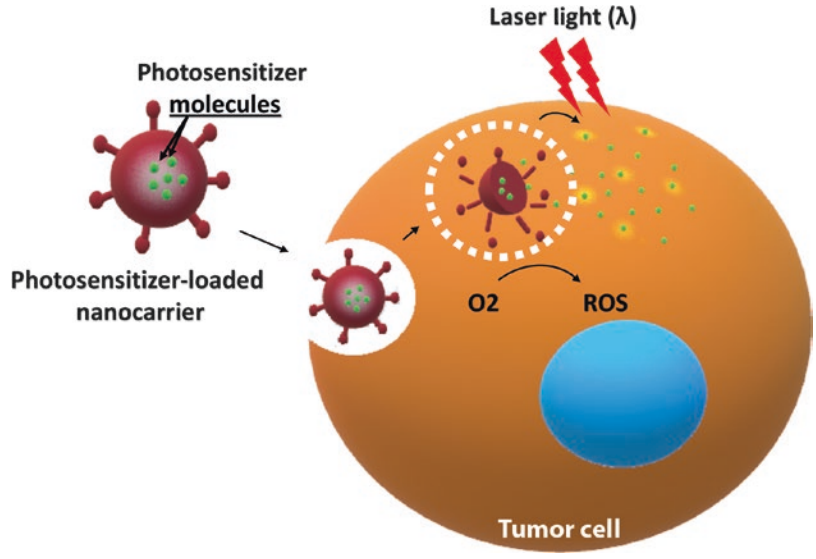

Fig. 13.4 Nanoparticles in photodynamic therapy. Nanoparticles can be used in PDT due to the delivery of light-activatable chemicals, known as photosensitizer molecules, to tumour cells. After the absorption of light, photosensitizer molecules can generate cytotoxic oxygen-based reactive species that cause cellular damage and cell death via oxidative stress

Table 13.4 Nanoparticles involved in PDT for the treatment of GC

Type of nanoparticle	Use	Photodynamic therapeutic (PDT) agent	Stage of development	References
PEG-GEL/PLA NPs	Carrier	CHA2HB	In vitro	[122]
PVP-coated rGO NPs	Carrier	Ce6	In vitro	[123]
Linolenic acid-conjugated polyhedral oligomeric silsesquioxane nanohybrids	Carrier	PPIX	In vitro	[124]
Upconversion LNPs	Carrier	PPIX	In vitro	[125, 126]
Semiconductor Pdots	Carrier	Tetraphenylporphyrin	In vivo/in vitro	[127]

PLA NPs are highly effective for the delivery and phototoxic enhancement of CHA2HB against cancer cells in vitro.

Another study successfully developed a facile surface functionalization strategy using chemically reduced graphene oxide (rGO) as a carbon NP model to allow both biocompatibility and receptor-targeted drug delivery [123]. To improve the aqueous dispersibility and biocompatibility of rGO and provide anchoring sites for RGD peptide, NPs were coated with polyvinylpyrrolidone (PVP). The nanodelivery system rGO-PVP-RGD could effectively load aromatic PS chlorin e6 (Ce6) via hydrophobic interactions and π-π stacking, as well as significantly increase the accumulation of Ce6 in the MGC803 GC cell line, improving the efficacy of PDT compared with Ce6 alone.

The poor water solubility and cellular internalization of protoporphyrin IX (PpIX), a PS used in PDT for cancer, limit its direct application. To improve this limitation, a biocompatible PpIX/linolenic acid-conjugated polyhedral oligomeric silsesquioxane (PPLA) nanohybrid was developed [124]. This nanocarrier system enhanced the intracellular uptake of PSs due to improved water solubility when evaluated using a human GC cell line (MKN-28).

Some studies describe the improvement in PDT efficiency against a human GC cell line (MKN45) using lanthanide NPs (LNPs) and 5-aminolevulinic acid (ALA) [125, 126]. In fact, PpIX is a PS that is selectively accumulated in cancer cells after oral administration of ALA. However, the low tissue penetrability resulting from blue incident lights required to excite PpIX limited its application to surface cancers. To overcome this limitation, these studies have shown how LNP could be employed as a light energy upconverter, which, upon irradiation with highly penetrative near-infrared (NIR) radiation, emits visible light to allow PpIX sensitization. An intermittent NIR irradiation of MKN45, pretreated with LNP and ALA, caused cell destruction.

A study describes the energy-transfer amplified singlet oxygen generation in semiconductor polymer dots (Pdots) for in vitro and in vivo PDT to treat GC [127]. Hydrophobic PS tetraphenylporphyrin was doped in the NPs. The antitumour effect of the Pdots was evaluated in vivo and in vitro. The in vitro studies showed that cancer cells were efficiently destroyed at a very low dose of the Pdots. Human gastric adenocarcinoma mice xenografts were significantly inhibited and eradicated in vivo.

Photothermal Therapy

Thermal therapy is a type of cancer treatment that involves heating of the tumour using radiofrequency (RF), microwaves, magnetic fields, or ultrasounds to cause irreversible cellular damage by loosening membranes and denaturing proteins, which ultimately kills cancer cells. However, thermal therapy has been limited by damage caused to surrounding normal tissue [128]. To overcome this problem, photothermal therapy (PTT) uses photothermal agents to achieve more controlled and selective heating of the target area, reducing thermal damage to the tumour (Fig. 13.5).

To be effective, photothermal agents need to have an enhanced light absorption and an efficient light-to-heat conversion. Traditional

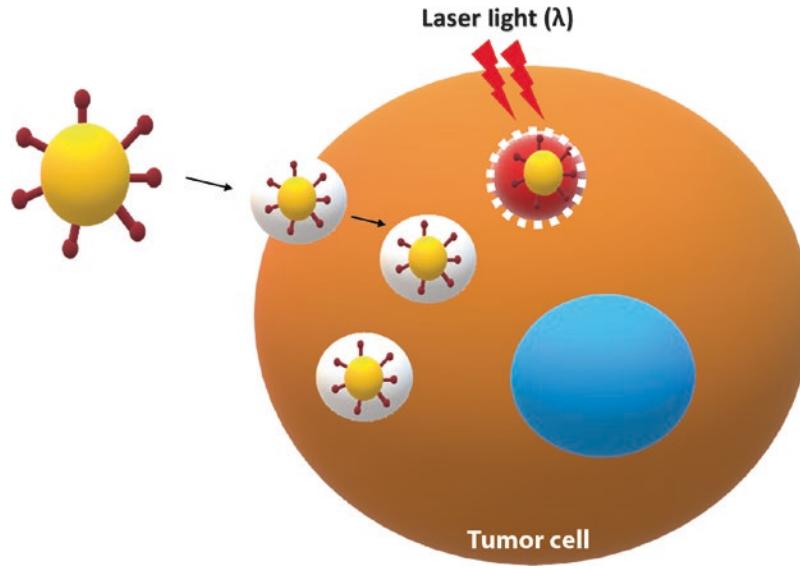

Fig. 13.5 Nanoparticles in photothermal therapy. Due to their efficient light-to-heat conversion, nanoparticles can be used in photothermal therapy. After the absorption of light, nanoparticles cause localized destruction. The controlled and selective heating of nanoparticles allows thermal damage of the target area while minimizing any damage to the surrounding normal tissue

Table 13.5 Nanoparticles involved in PTT for the treatment of GC

Type of nanoparticle	Use	Photothermal therapy (PTT) agent	Stage of development	References
GNR loaded-iPS-treated MMC	Photothermal agent	GNRs	In vivo	[130]
GNRs	Photothermal agent	GNRs	Ex vivo	[131]
Hb NPs	Carrier	Near-infrared dye IR780	In vivo	[132]
GO NPs	Photothermal agent	GO NPs	In vitro	[133]

agents suffer from low absorption, or external dyes (i.e. indocyanine green), have photobleaching limits. However, to overcome these problems, noble metal NPs (i.e. gold nanospheres, nanorods, nanoshells, and nanocages) have been developed because they have strong absorption in NIR regions of the electromagnetic spectrum due to surface plasmon resonance (SPR) [129]. This feature is advantageous because it increases the depth penetration of light into biological tissues, which exhibit minimal light absorption in this range. Table 13.5 shows a list of some NPs involved in PTT for the treatment of GC.

Gold nanorods (GNRs) have attracted substantial interest in PTT applications in the treatment of GC. In fact, despite their intrinsic tumour tropism ability, human-induced pluripotent stem cells (iPSs) are impeded in clinical applications of cancer therapy due to the formation of teratomas and their survival in normal organs. A team of researchers loaded GNRs with iPSs treated with mitomycin C (MMC) to suppress iPS proliferation as a safe delivery approach for the targeted photothermal treatment of GC [130]. The tumour cells were efficiently killed by the heat generated from the GNRs, and the iPS cells ultimately died due to the action of MMC 7 days after the photothermal treatment.

Another study aimed to find the optimal GNR concentration and laser power for inducing hyperthermic effects in tissues and test this effect on human oesophagogastric adenocarcinoma [131]. After irradiation with NIR light, a significant increase in temperature was measured in tissues incubated with an optimal concentration of

GNR solution compared with tissues not exposed to any GNRs. Thus, this method could be effective for irreversible photodestruction of oesophagogastric tumours, with minimal collateral damage expected in (healthy) tissues free from GNRs.

In contrast, a study used haemoglobin (Hb) NPs to improve the oral bioavailability of IR780, for in vivo antitumour application in PTT [132]. The HbNPs showed high stability in enzymatic and acidic conditions similar to the gastric environment and enhanced absorption of IR780 into the blood with accumulation at tumour sites. Thus, HbNPs represent a promising delivery system for improving the oral absorption of PS dye that could effectively cause a photothermal effect, resulting in tumour ablation after oral administration in tumour-bearing mice and defining new treatment modalities in GC.

Carbon-based nanomaterials have also been used in PTT for GC, particularly NPs based on graphene oxide (GO NPs). The ultrafast reduction of GO NPs with a femtosecond laser beam has been shown to create extensive microbubbling, which produces a microcavitation effect that introduces localized mechanical damage. Taking advantage of this phenomenon, GC cells labelled with GO NPs were irradiated with the laser, and the microbubbling effect greatly facilitated the destruction of cancer cells [133].

Nanoparticles for GC Diagnosis

As mentioned previously, most GC patients are diagnosed at an advanced stage. Therefore, it is difficult to cure this disease. Apart from early diagnosis, tumour staging, planning for surgical resection, and prognosis are also needed in clinical practices. In addition, GC is classified as a "localized tumour" that is slightly different from "systemic" tumours, such as breast cancer and lung cancer. "Locoregional metastasis" is the most important negative prognostic factor in GC [134–136].

Thanks to the unique properties that appear at the nanoscale, nanomedicine provides many benefits in the diagnosis of cancer [137–141]. Thereby, the "diagnosis" of GC using NPs includes the following:

1. NPs for systemic imaging
2. NPs for locoregional imaging
3. Surface-enhanced Raman spectroscopy (SERS) NPs for early detection
4. NPs used in the detection of GC-related biomarkers
5. NPs used in the detection of circulating tumour cells (CTCs)

Table 13.6 shows some studies that have made progress in these fields.

Nanoparticles Used in the Systemic Imaging of GC

Conventional imaging of GC, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission computed tomography (PECT), single-photon emission computed tomography (SPECT), and PET-CT, uses imaging contrasts and tracers for the whole body scan. However, these agents suffer from a non-specific distribution throughout the body, rapid clearance, not optimal pharmacokinetics and undesirable side effects [86, 142–144]. To overcome these limitations, a variety of NPs have emerged to enhance the imaging modalities of cancer due to many advantages, including nanoscale sizes, high agent loading, tailorable surface properties, controllable release patterns, and the EPR effect [86, 142], yet the successful development of safe and effective NP-based imaging modalities for in vivo and targeted GC imaging remains a great challenge.

In general, inorganic NPs are used as CT/MRI contrasts. Super paramagnetic NPs are the most studied agents [145]. There have been limited reports about other inorganic NPs.

The overexpression of CD146 in aggressive gastric or gastroesophageal cancer cells makes it an important biomarker for early diagnosis of GC [146, 147]. This biomarker has been used to engineer successfully NPs for molecular imaging of GC that could be applicable in tumours for

Table 13.6 Nanoparticles used in GC diagnosis

Imaging modalities	Types of nanoparticles	Targeting strategy	Application field	Stage of development	References
MRI	Dextran iron oxide NPs	Trastuzumab	Systemic imaging	In vivo/in vitro	[152]
	Liposome-coated fluorescent magnetic NPs	Trastuzumab	Systemic imaging	In vitro	[153]
	SPIO NPs	Passive targeting	Locoregional imaging	Phase I Clinical Trials	[161]
NIR fluorescence imaging	Nanocolloid ICG	Passive targeting	Locoregional imaging	Phase I Clinical Trials	[163]
	Liposomal ICG	Passive targeting	Locoregional imaging	In vivo	[164]
MRI/NIR fluorescence imaging	SPIO NPs coated with SiO_2	Anti-CD146 MAb	Systemic imaging	In vivo/in vitro	[148]
	Fluorescent magnetic NPs	BRCAA1 MAb	Systemic imaging	In vivo/in vitro	[114]
CT	Silica-capped gold nanoclusters	FA	Systemic imaging	In vivo	[156]
SPECT	^{111}In-labelled polymeric micelles	GRP78	Systemic imaging	In vivo	[157]
Upconversion luminescence imaging	PEGylated upconversion NPs	MGb2 Antibody	Locoregional imaging	In vivo	[160]
Multispectral optoacoustic tomography	PEGylated liposome-ICG	MUC-1 MAb	Locoregional imaging	In vivo	[165]
SERS-based CT	Magnesium sulphate aggregated silver NPs	Circulating RNA	Biomarker detection	In vitro	[181]
SERS-based biosensor	PNA probed-gold NPs	Ct DNA	Biomarker detection	In vitro	[185]
SERS-based endoscopy	SERS -NPs	EGFR and HER-2	Biomarker detection	In vivo	[186, 187]
Microfluidic biosensor	QDs	CEA, CA125 and HER-2/Neu	Biomarker detection	In vitro	[216]
Video capsule simulator	NIR labelled NPs conjugated to A1AT-specific antibodies	A1AT	Biomarker detection	In vitro	[217]
Ultrasensitive electrochemical nanobiosensor	Gold-magnetic nanocomposite	miR-106a	Biomarker detection	In vitro	[218]
Microfluidic biosensor	TiO_2 NP	CTCs	Biomarker detection	In vitro	[230]

image-guided therapy and surgery. For this purpose, superparamagnetic iron oxide NPs (SPION) were coated with nano dense-silica ($d$$SiO_2$) as core-shell NPs [148] and labelled with a near-infrared fluorescence (NIRF) dye and anti-CD146 MAb for a magnetic resonance (MR)/NIRF imaging study in the MKN45 xenograft GC model. The tumours were imaged at 30 min postinjection. This is the first successful study of functional NPs for MR/NIRF imaging of the cell surface glycoprotein CD146 in a GC model, suggesting that this system will be applicable in tumours for image-guided therapy/surgery.

BRCAA1 protein is found to be overexpressed in approximately 65% of GC tissues [114]. A study predicted that BRCAA1 could be one of

the potential targeting molecules for in vivo GC cells and aimed to develop BRCAA1v MAb-conjugated fluorescent magnetic NPs for in vivo targeted imaging of GC [114]. In comparison to pure fluorescent magnetic NPs, BRCAA1-conjugated fluorescent magnetic nanoprobes showed very low toxicity, lower magnetic and fluorescent intensities. They could be endocytosed by GC MGC803 cells and could target in vivo GC tissues with a diameter of 5 mm at 0.5 h and 12 h postinjection. Thus, they could be used to image GC tissues by fluorescence imaging and magnetic resonance imaging as well as local thermal therapy of early GC in the near future.

Overexpression of HER2, a tyrosine kinase receptor, is correlated to metastatic GC. Trastuzumab (Herceptin), a humanized MAb that is targeted to the extracellular domain of HER2, is used to treat this subtype of metastatic GC and is validated by the FDA [149–151]. Different studies in breast cancer have conjugated trastuzumab to different superparamagnetic NPs for imaging [149], such as dextran iron oxide NPs [152] and liposome-coated fluorescent magnetic NPs [153]. These approaches can also be used for HER2 overexpressing GCs [149, 154, 155].

Folic acid (FA) is one of the micronutrients required for normal human growth. FA depletion/deficiency has been linked to GC. A study was designed to produce FA-conjugated silica-capped gold nanoclusters/nanoprobes for dual mode fluorescent imaging and CT imaging [156]. This system was successful: it was biocompatible and could target the FA (+) MGC-803 cells in vitro and in vivo, demonstrating excellent red-emitting fluorescence and CT imaging.

For the nuclear imaging of GC, there have been some attempts to enhance the effective use of NPs. An endoplasmic reticulum protein, glucose-regulated protein 78 (GRP78), is a GC biomarker due to its overexpression on the GC cell surface. In tumour cells, GRP78 functions as a refolding protein, which translocates to the plasma membrane to protect cells from apoptosis. A study designed GRP78 binding peptide (GRBP)-guided ^{111}In-labelled polymeric micelles for nuclear imaging detection of tumours [157]. In vivo studies in a murine GC xenograft demonstrated that the radioactive intensity measured in animal tumours treated with GRP78BP polymeric micelles was statistically higher than in animals administered untargeted micelles, demonstrating that GRP78BP could enhance the accumulation of micelles to the tumour tissue and could be useful in the application of nuclear imaging for tumour diagnosis.

Nanoparticles Used in Locoregional Imaging of GC

GC is specifically characterized to be a "localized" disease with lymphatic and peritoneal metastasis as independent prognostic factors [158, 159]. The status of both lymph node (LN) and peritoneal dissemination is extremely helpful in the pretreatment stage for proper treatment planning. However, there are limited imaging modalities applicable in these areas [160], such as MRI [161], upconversion luminescence imaging [162], NIR fluorescence imaging [163, 164], and multispectral optoacoustic tomography [165].

Diagnosis of LN metastasis in GC is essential to direct the operative approach and is performed through imaging tests, such as CT and ultrasonography (US). However, the accuracy of such tests has not been adequate. Ferumoxtran-10 is a lymphotropic contrast agent for MRI with reported efficacy for the detection of metastatic LNs in various cancers. To investigate the efficacy of ferumoxtran-10-enhanced MRI for the diagnosis of metastases LN in GC, a study enrolled 17 consecutive patients who were diagnosed with a non-early stage of GC. All the patients were examined by MRI before and 24 h after IV administration of superparamagnetic iron oxide—ferumoxtran-10. LNs were more readily identified and diagnosed by MRI images 24 h after IV administration of superparamagnetic iron oxide—ferumoxtran-10, suggesting that ferumoxtran-10-enhanced MRI is useful in the diagnosis of metastatic LNs and that the use of this modality will be helpful to direct the treatment course for GC patients [161].

Another type of molecular imaging probe using core@shell-structured NaGdF4:Yb,Er@NaGdF4 upconversion NPs coated with polyethylene glycol (PEG) has high sensitivity for the detection of lymphatic metastasis of GC [160]. These NPs improved the detection sensitivity in imaging by displaying a satisfactory signal-to-noise ratio. Lymphatic metastases smaller than 1 mm were successfully detected in a mouse model of human GC [162], revealing the important role of these NPs in a highly effective approach for regional GC diagnosis.

A new modality for cancer imaging aiming to identify tumour and regional metastases during surgical resection is called image-guided surgery, a real-time imaging technique that is very useful for the surgical planning of GC and may be optimized to provide a higher signal to background by the incorporation of fluorescent NPs [166]. For instance, indocyanine green (ICG), which is an FDA-approved fluorescent probe, has shown important potential in image-guided surgery for GC. Several types of fluorescent NPs in phase I trials have been reported, such as nanocolloid ICG [163], liposome-embedded ICG [165, 167], HA-derived ICG NPs [168], SPIO-phospholipid-PEG-ICG [169], and others.

A study on 22 GC patients, using ICG:Nanocoll (adsorption of ICG to a nanocolloid), showed that among 21 patients with at least 1 LN detected by NIR fluorescence imaging, 8 had tumour-positive LNs. This technique offers 90% accuracy [163]. NIR-fluorescent liposomal probe LP-ICG-C18, a synthesized ICG liposomal derivative, has been used to evaluate the peritoneal metastases of GC on nude mice. It can effectively target peritoneal disseminated tumours and possibly detect them by a NIR imaging system [164]. In addition, ICG-lactosomes [170] (NPs composed of poly(L-lactic acid)-based depsipeptide [171]) and ICG-PEGylated liposome-ICG [165] are also used in the diagnosis of peritoneal dissemination of GC for theranostic purposes.

NPs have also been used in the field of US, such as nanobubbles, which were used as an US contrast agent, showing an improved contrast imaging effect on GC xenografts [172]. They were able to pass through the gaps between endothelial cells in the tumour vascular system to reach the tissue space.

Nanoparticles Used in Surface-Enhanced Raman Spectroscopy for the Early Detection of GC

The detection of GC at early stages is very challenging. It requires specific biomarkers at very low concentrations with high selectively to elude false positives. In addition, conventional white-light endoscopy is the most important and effective diagnostic method for the early detection of GC, apart from occult blood tests of stools [173]. However, it offers only structural information for the gastrointestinal tract without biochemical information. Among the most promissory methods to detect biomarkers using nanotechnology and to enhance the sensitivity of endoscopy, we will highlight herein those based on metallic NP plasmons.

The collective oscillation of the conduction electrons is called the plasmon [174]. Two types of surface plasmons exist: those coming from a bulk metal and those coming from metallic NPs. The metallic NPs have localized surface plasmons. Their excitation induces optical properties that are hardly achievable in other optical materials [175]. Thus, they can be used for analytical purposes, such as plasmon-enhanced spectroscopy: enhancing the signals of Raman scattering and fluorescence spectroscopy [176]. These materials can be synthesized and modified to be conjugated to antibodies, ligands, and drugs to yield a wide range of applications in many fields, such as biotechnology, magnetic separation, preconcentration of target analytes, targeted drug delivery, and vehicles for gene and drug delivery, especially diagnostic imaging [177].

Several imaging modalities have been developed as an aid to image GC, and the most important is SERS. The detection of biomarkers with very high sensitivity plays a crucial role in GC diagnosis. Gold and silver NPs can control and manipulate light at the nanoscale. They have a localized surface plasmon, so they can act as optical antennas that capture and radiate light to their vicinity, enhancing the Raman signal of

many folds. Under favourable conditions, this technique can detect single molecules [178–180]. Discovered since more than 40 years ago, SERS is now a powerful analytical tool for molecular detection and characterization. In recent years, a great deal of interest has been focused on the development of SERS biomedical applications. However, the lack of the fabrication of reproducible nanostructures and their instability and tendency to aggregate have been serious obstacles to the practical applications of SERS. Nevertheless, aggregated NPs function as hotspots where the electromagnetic enhancement is particularly high, enhancing the Raman signal. Thus, controlling the formation of the aggregation in a way as to prevent total collapse of the colloid becomes a main task for experimental SERS.

Few applications have been reported in GC. A study developed a useful clinical tool for the noninvasive screening and detection of cancer with good diagnostic sensitivity and specificity. This method is based on SERS technology to analyse RNA in the circulation to discriminate GC patients from healthy controls [181]. RNA was extracted from serum samples and then scanned for significant differences in the spectral bands. Silver NPs partially aggregated using magnesium sulphate and were used as the SERS-active substrate presenting strong SERS signals to RNA. Many other papers were published concerning this method [173, 182, 183]. Despite significant differences in the intensity of several bands between cancer patients and healthy volunteers, there were no significant frequency shifts. Thus, it becomes hard to interpret with certainty the meaning of the spectral changes. This approach may serve as a screening method before performing further diagnostic tests.

The surface of the NPs is composed of two components: one component is an antibody that endows them with more specificity for their target, and the other component, called the Raman reporter, is easily detectable with Raman spectroscopy. Thanks to the functionalization of NPs, many studies have been reported on the use of plasmon-enhanced methods, in which better targeting and less aggregation can be achieved even though the synthesis process becomes more complicated and expensive [184]. These applications have been used for different types of cancer but not for GC. The development of a very sensitive detection method is highly likely using this strategy.

Circulating tumour DNA (ctDNA) is a double-stranded DNA that represents a promising biomarker for noninvasive cancer diagnosis [185]. Mutations at two hotspots of E542K (G70271A in exon 9) and E545K (G70282A in exon 9) and methylation of ctDNA of *PIK3CA* (phosphatidylinositol 3-kinase catalytic subunit) are tumour-specific genetic and epigenetic markers of ctDNA, respectively, that are well-known in many types of cancers including breast, colon, brain, liver, stomach, and lung [185]. Therefore, a study developed a peptide nucleic acid (PNA)-based nanoplasmonic for biosensor dual biomarker detection [185]. To detect genetic markers of ctDNA, gold NPs were coupled to PNA that captures and binds specifically to ctDNA. It results in a change in the refractive index surrounding the biosensor surface, generating a distinct localized surface plasmon resonance (LSPR) peak shift in the Rayleigh light scattering pattern. For epigenetic markers of ctDNA detection, gold NPs (immunogold colloids) coupled with methylcytosine MAb that bind specifically to methylated CpG sites on the ctDNA sequence were used. The tumour-specific genetic and epigenetic markers of ctDNA were successfully detected, and the enhancement assay increased the signal reducing four times the limit of detection (LOD).

This sensitive and multiplexed platform detected ctDNA in clinical samples at a low concentration. However, compared with the more commonly used methods, such as high-performance liquid chromatography (HPLC) or absorption spectroscopy, these techniques are still expensive, but they carry a huge analytical potential. Therefore, related approaches are starting to be evaluated in clinical settings. In contrast, to enhance the sensitivity of endoscopy, a study [173] has reported a noncontact, fibre optic-based Raman spectroscopy device that has the potential to provide real-time, multiplexed functional information during routine endoscopy. This tool was adjusted to be appropriate for the detection of functionalized SERS NPs as molecular imaging contrast agents.

When conjugated to tumour-targeting ligands, SERS NPs will target tumour biomarkers and be detected using a Raman spectroscopy device [173, 184]. As a result, a certain subgroup of cells (e.g. cancer cells) can be detected. A study [186, 187] used anti-EGFR-MAb and anti-HER2-MAb SERS NPs on the luminal surface of rat oesophagus and found that the EGFR and HER2-expressing tumour cells were precisely located and that the visualization and quantification of biomarker expression were in agreement with the immunohistochemistry and flow cytometry validation data. This technique is clearly based on one or more molecules expressed on the surface of cancer cells with high specificity. For GC, such molecules include carcinoembryonic antigen (CEA), cancer-related antigen 19-9 (CA19-9), cancer-related antigen 72-4 (CA72-4), HER2, EGFR, and others. Although none of the above-mentioned markers have 100% sensitivity and specificity, the combination of routine optical endoscopy and SERS NPs will probably provide a more sensitive method for early GC detection.

Nanoparticles Used in the Early Detection of GC-Related Biomarkers

The availability of GC-related biomarkers plays an important role in the early diagnosis of disease; they offer particular properties in turn, and they could be exploited for the development of nanostructured biosensors to increase high analytical performances [188].

Certain types of NPs may be used to increase the sensitivity of a biosensor and to be more accurate, precise, and faster [188]. The application of NPs could be advantageous in optical-based nanosensors [189–192], electrical- or electrochemical-based nanosensors [188, 193–195], fluorescence-based nanosensors [196], and magnetism-based nanosensors [197–199]. Several NPs are used in nanosensors, such as gold, magnetic NP-based biosensors, and quantum dots (QDs), which are the mostly used [189, 200–206]. These NPs have been successfully used into the nanosensors for the detection of carcinoembryonic antigen (CEA) [207–210], cancer antigen 125 (CA125) [200, 211, 212], CA724 [213], and HER2 [214]. In addition, these NPs could be useful for the exploration of new biomarkers for the early detection of GC [215].

Other nanostructures, such as nano-biochips, have also been applied in this field with NPs [216]. The integration of semiconductor NP QDs into biosensors in serum and saliva led to an amplified signal of CEA, CA125, and Her2/Neu biomarkers. In that study, QD probes were used in this biosensor format to produce a signal 30 times greater than that of standard molecular fluorophores with an approximately twofold reduction in observed limits of detection relative to the enzyme-linked immunosorbent assay (ELISA). A study reported a platform with immunoassay capabilities comprising sensing and detecting compartments serving as a real-time diagnostic for the early detection of a secreted biomarker model α1-antitrypsin precursor (A1AT) of GC [217]. The detecting compartment embeds NIR fluorescently labelled NPs conjugated to A1AT-specific antibodies. The specific recognition reaction between the captured A1AT and the immuno-NPs generates profound fluorescence with an intense signal that is sufficient to be detectable by a conventional endoscope or a video capsule. Another study used a double-specific probe methodology and a gold-magnetic nanocomposite as a tracing tag to develop an ultrasensitive electrochemical nanobiosensor for the detection of miR-106a, an overexpressed microRNA in GC [218]. The results showed many advantages, including high specificity, remarkable selectivity, compliant storage stability, and great performance, in an investigation of real samples with no pretreatment or amplification, suggesting that this miRNA-nanobiosensor can be used for clinical early detection of GC and additionally to screen any miRNA sequence.

Nanoparticles Used in the Detection of Circulating Tumour Cells (CTCs) of GC

CTCs are cancer cells, deriving either from primary tumours or from metastatic sites, which

separate from the tumour and circulate in the peripheral blood to cause possibly metastasis [219]. CTCs found in the blood have been investigated as potential biomarkers for the diagnosis, prognosis, and molecular testing of metastatic GC [220, 221]. The term "liquid biopsy" is related to the identification of CTCs in the peripheral blood of cancer patients. Currently, both circulating nucleic acids and extracellular vesicles are included in the term liquid biopsy. Compared with tissue biopsies, liquid biopsies can be associated with noninvasiveness and a real-time response by offering the ability to guide the choice of therapy.

However, it is challenging to develop a CTC detection method with clinically significant specificity and sensitivity. Advances in nanotechnology and new nanomaterials offer promising enhancement for the detection of CTCs [220, 222], such as NPs [220, 223], microfluidic chips [222, 224], nano-roughened structures [222], nanoVelcro chips [219], and nanofibres [225].

The NPs used for the detection and/or isolation of CTCs usually include two parts: ligands that specifically bind to a known biomarker for CTCs (e.g. antibodies, aptamers) and NPs that can produce a specified signal and can be detected out of the blood [226]. Some NPs, including gold [227], magnetic [228, 229], QDs [226], and TiO_2 [230], are capable of detecting CTCs from blood. Interestingly, some of these NPs can simultaneously detect and isolate CTCs [229].

In a recent study [227], the use of magnetic NPs in combination with gold-plated carbon nanotubes enabled the detection of CTCs from a large volume of blood in the vessels of tumour-bearing mice. However, only a few reports on the detection of gastric CTCs by NPs are available. The isolation of CTCs from peripheral blood of GC patients with a nano-film made of TiO_2 biocompatible NPs has been reported, and 50% of the captured cells detached from the substrate and could be expected for potential clinical use [230]. Other NPs have emerged to detect special markers of GC or GC stem cells and are expected to be potential candidates for CTC detection, such as CD133 [231], HER2 [153], CD44 [232], and CD146 [146, 148].

Nanoparticles as Theranostic Agents

The word "theranostics" is defined as the combination of diagnosis with therapy. Various nanocarriers of biodegradable polymers are applied to sustain, control, and target the co-delivery of diagnostic and therapeutic agents for more effective theranostics and reduced side effects, including polymer conjugates, micelles, liposomes, dendrimers, metal, inorganic NPs, and carbon NPs. The importance of these platforms lies in the diagnosis and treatment of the diseases at different tumour stages, when they are most likely curable or even not curable [233]. The diagnosis and delivery of therapy to targeted cells are enhanced theranostic nanomedicine by coupling a targeting ligand and biomarkers [234]. NPs ranging from 10 to 1000 nm in size are used in theranostic nanomedicine. They are composed of different macromolecules or polymers that are conjugated to diagnostics and therapeutic agents for simultaneous diagnosis and treatment at cellular and molecular levels [235]. The advantages of this platform include controlled release, targeted delivery, and a higher transport efficiency by endocytosis [236], as well as the induction of stimulus-responsive agent release [237]. The co-encapsulation of multiple diagnostics and therapeutic modes has allowed multimodality nanotheranostics and resulted in enhanced performances of their application [238].

Oral delivery of theranostic nanomedicine can enhance oral bioavailability. For instance, D-α-tocopheryl polyethylene glycol 1000 succinate-based nanomedicine was used for the oral delivery of chemotherapeutic agents [239].

The therapeutic agents in theranostic nanomedicine include hydrophobic organic drugs, proteins, peptides, and genetic materials. Diagnostic agents are also generally used in theranostic nanomedicine and include optical imaging agents, such as fluorescent dyes or QDs; magnetic resonance imaging agents, such as superparamagnetic metals; nuclear imaging agents, such radionuclides; and CT agents, such as heavy elements [234, 240, 241].

NPs used in theranostics are based on two strategies: first, NPs can be detected themselves

by imaging modalities, and second, they support the targeted co-delivery of diagnostic and therapeutic agents. These advanced theranostic platforms provide sensitive diagnosis platforms with accurate targeting and effective delivery of materials [242].

PEG-coated Fe_3O_4 NPs were chemically produced by co-precipitation method in which PEG functions as a stabilizer and dispersant. Such Fe_3O_4 NPs with tunable magnetic properties and a favourable size have shown promising biomedical applications [243]. In GC theranostics, polyethylene glycol-grafted polyethylenimine functionalized with superparamagnetic iron oxide NPs (PEG-g-PEI-SPION) has been successfully applied.

A study using an antibody-directed non-viral vector, combining PEG-g-PEI-SPION and a GC-associated CD44v6 single-chain variable fragment ($scFv_{CD44v6}$-PEG-g-PEI-SPION), was constructed for a GC-targeting and magnetic resonance imaging (MRI)-visible nanocarrier for siRNA delivery [244]. The cell viability and siRNA transfer efficiency were effective in vitro using the human gastric carcinoma cell line SGC-7901. Fluorescence-based imaging techniques revealed the success of the cellular uptake and distribution of NPs complexed with siRNA. Additionally, the targeting efficiency against GC was verified in vivo in nude female mice by MRI and by histology analysis, revealing the crucial and promising role of the non-viral vector $scFv_{CD44v6}$-PEG-g-PEI-SPION in gene therapy and the diagnosis of GC [245].

Another study developed FA- and disulphide (SS)-polyethylene glycol-conjugated polyethylenimine complexed with the SPION (FA-PEG-SS-PEI-SPION) polyplex for siRNA delivery system for programmed death ligand-1 (PD-L1) knockdown. PD-L1 is highly expressed in GC, interacts with PD-1 receptor on T cells, and is involved in T-cell immune resistance. The characterization of FA-PEG-SS-PEI-SPIONs determined its high binding ability, minimal cytotoxicity, higher transfection efficiency, and cellular internalization of the system in the folate receptor-overexpressing GC cell line compared with a non-FA-conjugated polyplex. In diagnostics, the polyplex functioned as a T_2-weighted contrast agent for enhancing cancer MRI. At the cellular level, one of the four PD-L1 siRNAs showed effective PD-L1 knockdown in cells overexpressing PD-L1, demonstrating changes in secreted cytokines and highlighting the potential of this class of these multifunctional theranostic NPs for the treatment and diagnosis of GC [246].

PEG-coated Fe_3O_4 NPs were also used as a miRNA delivery system to modulate the drug resistance of GC cells by enhancing miRNA-16 (miR16) expression in SGC7901/ADR cells. MiR16 plays a crucial role in reducing the drug resistance of SGC7901 cell lines to Adriamycin (ADR). ADR-induced apoptosis of SGC7901/ADR was examined by MTT and TUNEL and showed that miR16 combined with PEG-coated Fe_3O_4 NPs treatment increased cell apoptosis in vitro significantly. MiR16 and PEG-coated Fe_3O_4 NPs were able to significantly suppress SGC7901/ADR tumour growth in SGC7901/ADR(fluc) tumour-bearing nude mice, possibly by increasing SGC7901/ADR cell sensitivity to ADR. This system suggested an efficient delivery of miR16 by PEG-coated Fe_3O_4 NPs for drug-resistant tumours [247].

A different effective strategy for drug delivery using polymer-coated magnetic carriers can both increase drug utilization and reduce adverse reactions. Using these carriers, sensitivity to physical stimuli, such as a magnetic field and pH, has been developed, and the drugs have been conjugated to magnetic particles to target the desired position [248]. A type of magnetic-polymer nanocarrier was attached to folate receptor targeting and pH-sensitive multifunctionalities to carry doxorubicin (DOX) for advanced GC (AGC) treatment. Folate-coupled, pH-sensitive, amphiphilic poly(β-aminoester) self-assembled with hydrophobic oleic acid-modified iron oxide NPs, resulting in hydrophobic interaction area, as a reservoir for lipophilic DOX (F-P-DOX). Using confocal microscopy, it was shown that F-P-DOX treatment could maintain higher DOX accumulation in cells than P-DOX without folate conjugation, leading to an increased efficiency of DOX internalization at pH 6.5 than at pH 7.4. Using electron microscopy and real-time polymerase chain reaction, superior

efficacy of F-P-DOX than free DOX on GC was observed. The efficacy was also determined by the MTT assay and xenograft model. Moreover, the accumulation of F-P-DOX in the tumour region was detected by MRI. Together, these observations affirm F-P-DOX as a promising theranostic candidate for AGC treatment [249].

Similarly, magnetic alginate (Alg)-CH beads were loaded with albendazole (ABZ) to test their pH sensitivity and drug release characteristics. These magnetic beads showed unique pH-dependent swelling behaviours with continuous release of ABZ. The beads also showed the magnetometer measurement data, a superparamagnetic property, as well as a fast magnetic response, revealing that they might be successfully used as a magnetic drug targeting system for ABZ in the gastrointestinal tract [248]. Another successful pH-sensitive magnetic NP (MNP) for targeted anticancer drug carriers is obtained by coating MNPs with poly(acrylic acid) (PAA) to obtain PAA@MNPs. These NPs exhibit a small size within 100 nm, good stability, and superparamagnetic properties. DOX was loaded onto MNPs (MNPs-DOX) via electrostatic interactions, with good drug-loading content and efficiency. Release studies showed that MNPs-DOX had excellent pH sensitivity, with 75.6% of the loaded DOX released at pH 4.0 in 48 h. MTT assays using the HUVEC and MCF-7 cell lines (breast cancer) demonstrated that MNPs-DOX had high antitumour activity, while the PAA@MNPs were practically nontoxic. Thus, PAA@MNPs would be a candidate for biomedical applications in GC, and MNPs-DOX could be used in targeted cancer therapy [250].

As described previously, PDT is a special theranostic modality for a number of diseases, based on the systemic, local, or topical administration of a nontoxic drug or dye known as PS followed by selective fluorescence characterized by an appropriate wavelength and intensity of light. The emitted light can be employed in photodiagnosis and molecular imaging to locate diseases, known as PS fluorescence detection (PFD). Online imaging of the drug for the detection of disease, image-guided drug delivery and treatment, guidance of surgical resection, and monitoring of treatment response can be performed by organic fusion of PFD and PDT [251]. A study examined PS-conjugated magnetic NPs with a diameter of ~20 nm designed for use in GC imaging and therapy, especially to integrate tumour targeting, imaging, and selective therapy into a small single NP (<50 nm). The Ce6 PS was covalently coupled onto the surface of magnetic NPs with silane coupling agent, allowing spectroscopic and functional properties for NIR fluorescence imaging and PDT and resulting in magnetically guided drug delivery and MRI. This platform is suitable for simultaneous targeting PDT and in vivo dual-mode NIR fluorescence imaging and MRI of nude mouse model with GC or other tumours [252] given the good stability and high water dispersibility and solubility, good biocompatibility, non-cytotoxicity, enhanced PS fluorescence detection, and remarkable photodynamic efficacy upon irradiation [251].

The theranostic properties of a new drug delivery system based on the loading of NPs with ICG derivative ICG-loaded lactosome (ICGm) were also applicable in a murine draining LN metastasis model of GC. The preoperative and intraoperative diagnoses of LN metastasis in patients with GC are important for the determination of the extent of LN dissection for the establishment of individualized treatment strategies. In vivo imaging successfully revealed metastatic LNs in the ICGm-treated but not in the ICG-treated mice. PDT using ICGm-induced apoptosis inhibited the growth of metastatic LNs, representing ICGm as a novel theranostic platform for LN metastasis of GC [165, 253].

ICG has also been strongly suggested for use as a photo-absorbent fluorescent probe that has been incorporated into clinically relevant PEGylated liposomes as a nanodevice for diagnosis. PEGylated liposome-ICG was synthesized using anti-MUC-1 humanized MAbhCTM01 as a tumour-specific theranostic system. Noninvasive tumour accumulation of these MAb-targeted liposomes was observed over time in a tumour mouse model using multispectral optoacoustic tomography (MSOT). Furthermore, both targeted and nontargeted liposome-ICG formulations preferentially accumulated in the tumour.

Table 13.7 Nanoparticles used in GC theranostics

Types of nanoparticles	Imaging strategy	Anticancer strategy	Stage of development	References
PEG-g-PEI-SPION	MRI	CD44v6 siRNA	In vitro/in vivo	[244]
FA-PEG-SS-PEI-SPIONs	MRI	PD-L1	In vitro	[246]
PEG-coated Fe_3O_4 NPs	MRI	MiRNA	In vitro/in vivo	[247]
pH-sensitive magnetic-polymer NPs with folate receptor targeting	MRI	DOX	In vitro/in vivo	[249]
PS-conjugated magnetic NPs	MRI	PDT	In vivo	[251, 252]
ICGm	NIR fluorescence imaging	PDT	In vivo, ex vivo	[253]
PEGylated liposome-ICG	MSOT	DOX	In vivo	[165]

A new study has reported the co-encapsulation of the natural herbal substances chrysin (Chr) and Cur in PEGylated PLGA NPs to explore their inhibitory effect against Caco-2 colon cancer cells. The free drugs and the nanoformulation showed dose-dependent cytotoxicity in Caco-2 cells. The nanoformulation had a more antiproliferative effect with induced growth arrest of cancer cells [254].

The high stability and sensitivity of a Gd(III) amphiphilic complex loaded in poly(lactic-co-glycolic acid) NPs (PLGA NPs) allowed their accumulation in vivo in a murine melanoma xenograft and demonstrate their promise as theranostic MRI agents, once loaded with drug and contrast agents [255].

Matrix metalloproteinase (MMP) 2/9, also known as gelatinases A/B, play a key role in cancer invasion and metastasis. The gelatinase-responsive copolymer (mPEG-PCL) was synthesized for anticancer drug delivery to make use of MMP2/9 as targets for drug delivery. The cellular uptake of GEL-NPs was correlated with the level of gelatinases, which also influenced the in vitro antitumour effect of GEL-NPs. The anticancer effect of GEL-NPs exceeded the DOC. The cytotoxicity study of primary lung cancer cells also confirmed the effectiveness of the GEL-NP targeting strategy [142]. This strategy could be applied to GC.

Finally, DOC-NPs based on the gelatinase-stimulus strategy were used to compare their radiosensitization efficacy with DOC in GC. DOC-NPs showed significant radioenhancement compared with DOC in all three gelatinase-overexpressing GC cells, associated with G2/M arrest enhancement, reactive oxygen species (ROS) production, and apoptosis induction. In addition, the radiosensitization efficacy of DOC-NPs was more prominent than DOC by intravenous injection in the xenograft. The gelatinase-mediated nanoscale delivery system serves as a potential strategy for radiosensitizer selectivity by manipulating the common microenvironment difference between the tumour and normal tissue [43].

Table 13.7 shows a list of some nanoparticles used as theranostic agents in GC.

Toxicity of Nanoparticles

NPs possess the capacity to revolutionize medical imaging, diagnostics, as well as therapeutics of GC. However, the toxicity of NPs should also be taken into consideration. Several studies have investigated the toxicity associated with specific NPs in GC. For example, a study has demonstrated that NPs made from copper increase hydrogen and bicarbonate ions and could damage gastric tissues [256]. Another study has demonstrated that high intake of superparamagnetic NPs can lead to the accumulation of iron in a specific organ to which it is delivered and thus produce toxic effects and lead to DNA damage [257]. Several studies focusing on three categories of nanomaterials, nanometals and metal oxides, carbon-based NPs, and polymer/den-

drimers, have demonstrated some toxic results of these NPs when used at a high dose. However, some in vivo studies have shown that a low dose of these NPs provides nontoxic results [258]. Platinum-based NPs show a strong response against GC cells but can still accumulate in the liver or spleen and show a cytotoxic effect. To overcome this problem, the incorporation of polymers, which are safe and easily biodegradable, could reduce the side effects of NP-based anticancer formulations, i.e. the use of hyaluronan in platinum nanoparticulate-based anticancer drugs [259]. Hence, short-term and long-term toxicity studies are also needed in both cell culture and living animal models before these agents can gain FDA approval for clinical trials.

Conclusions

Currently, cancer is still an alarming disease, and people panic when they hear about it. Like all other cancers, GC can lead to death, and it is mandatory to find new ways to address this disease as existing strategies are not sufficient. This chapter describes many different applications for which NPs are being used to fight against GC. By blending with existing treatment methodologies or creating new treatments, the use of NPs could pave the way to treat GC more easily than before. In addition, the use of NPs has increased the sensitivity and specificity of GC diagnosis modalities in systemic and locoregional imaging, led to early detection and identified biomarkers, and helped elucidating CTCs. However, there are some limitations of nanomedicine. A number of limitations that are shared among current NPs compromise their further transition into clinical use. These limitations are synonymous with the obstacles that researchers are currently trying to overcome [260, 261] and include immunogenicity, poor site-specific accumulation, production costs, inability to overcome barriers in the tumour microenvironment (high interstitial fluid pressure, interactions with collagen matrix), and inability to treat small metastases for which EPR is not evoked. By successively addressing each of these barriers, innovative design features can be rationally incorporated to create a new generation of NPs, achieving a paradigmatic shift in NP-based diagnosis and therapy.

Concerning GC treatment, unlocking the full potentiality of NPs as well as safely transferring there use into clinical trials, which would eventually lead to industrial-based production, requires further dedication and effort. Concerning GC diagnosis, the application of NPs is restricted due to the limits of the specificity of markers or ligands expressed by GC. Furthermore, a large number of studies follow similar study designs for the diagnosis of breast cancer, lung cancer, and colorectal cancer. Therefore, to promote the development of nanomedicine in the treatment and diagnosis of GC, further studies and increased collaboration and knowledge exchange between scientists are needed.

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V. Canzonieri, A. Giordano (eds.), *Gastric Cancer In The Precision Medicine Era*,
Current Clinical Pathology, https://doi.org/10.1007/978-3-030-04861-7