Saman Warnakulasuriya · Zakir Khan
Editors

Squamous Cell Carcinoma

Molecular Therapeutic Targets

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Springer

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ISBN 978-94-024-1083-9 ISBN 978-94-024-1084-6 (eBook)
DOI 10.1007/978-94-024-1084-6

Library of Congress Control Number: 2017931530

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The registered company is Springer Science+Business Media B.V.
The registered company address is: Van Godewijckstraat 30, 3311 GX Dordrecht, The Netherlands

Foreword

"**Squamous Cell Carcinoma: Molecular Therapeutic Targets**" is a refreshing deviation from the standard text books on Head and Neck cancer, currently available, which either deal with 'only basic science' or 'only clinical care'. The Editors, Drs. Saman Warnakulasuriya and Zakir Khan, have taken the bold step of taking a stab at combining, the frontiers in science and state of the art in clinical care, and compiling this hybrid and authoritative text on the molecular basis of current therapy in Head and Neck cancer.

The invited authors of each chapter are internationally recognized leaders in their own right who have carefully and comprehensively accumulated current information and presented it in a concise and systematic fashion, on the biopathology of Head and Neck cancer, role of cancer stem cells and the molecular oncology for the genesis and progression of squamous cell carcinoma. The second half of the book deals with therapeutics derived from the knowledge gained from laboratory research in the past two decades, and very succinctly presents, targeting p53, EGFR blockade, mTOR signalling, and the micro RNA as a biomarker, the current status of targeted therapies, approved for clinical care, and currently in investigational trials. The contributors also delve into the growing interest in immune modulators and provide an overall appraisal of targeted therapies in squamous cell carcinoma of the head and Neck.

This stimulating compendium presents an easy read and understanding of the state of the art in the application of breakthroughs in basic science translated to clinical care and supports the current philosophy of delivering personalized medicine to each and every patient, for best oncologic outcomes, minimal sequela of therapies, and optimal quality of life. Thus, it would be of great interest to the practicing clinician, the students and trainees as well as scientists, who have a common goal of remaining abreast with the state of the art and science in Head and

Neck cancer. This text book is an authoritative source in the subject and is highly recommended to clinicians, students, trainees and scientists, as well as libraries of medical schools, and postgraduate institutions.

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Preface

After several decades of profound advances in our understanding of the genetics and molecular biology of Head and Neck cancer, it has become clear that the growth and survival of cancer cells are intimately linked to and regulated by "cancer genes". Some genetic predispositions are inherited: most cancer genes develop as acquired changes to the host genome or epigenome from environmental influences. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules ("molecular targets") that are critical to the biochemical pathways controlling cell proliferation, and the growth and metastasis of cancers. Targeted therapies are currently the focus of much anticancer drug development. So far, few targeted cancer therapies have been approved by the Food and Drug Administration (FDA) in the USA to treat Head and Neck cancer. Others are being studied in Phase 2/3 clinical trials, and many more are being tested in vivo in animal models and in in vitro settings.

With chapters written by internationally renowned experts, this book should be useful for students studying cancer biology at all levels and an essential reference guide for clinicians involved in all aspects of the care of patients with Head and Neck cancer. It provides a ready source of the background science behind the many new agents they will come across in their daily practice. The chapters of the book are grouped in two sections; the first 4 contain introductory information that will be helpful to the clinicians while serving as reviews for scientists. Chapter 1 gives an essential overview—partly historical and also cutting edge—of the fundamental biological processes which become deranged in cancer. Chapters 2 and 3 complement the above with more detail of particular aspects of the behaviour of the malignant cell phenotype. We present in Chap. 4 the emerging, positive clinical data obtained to date on several molecular pathways that have rationale as targets for cancer therapy. The review of the literature outlines the extensive upstream and downstream regulatory crosstalk and molecular heterogeneity of Squamous Cell Carcinomas of Head and Neck (SCCHN). Taken together, these chapters provide the rationale for personalized medicine: the targeting of treatment to the individual patient's genetic abnormalities. The state of the art and science of such approaches are presented in Chaps. 5–9. Each focuses on a specific molecular target that is

currently in use or being developed for treating Head and Neck cancer. The final chapter captures the current status of drug development, particularly in clinical trials.

We intend the book to appeal to senior clinicians and academic trainees from all backgrounds who care for patients with Head and Neck cancers, in order to encourage them to join clinical trials and establish research collaborations. There is a great potential to improve the lives of patients with both early and advanced Head and Neck cancers, to test systemic therapies, perhaps as adjunctive treatments to established surgical and radiation-based approaches, or even as first-line treatment of otherwise incurable disease. In all of these approaches, we seek not only to prolong life, but also to minimize toxicity and maintain quality of life as best we can.

London, UK Saman Warnakulasuriya
California, USA Zakir Khan
December 2016

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Chapter 1
Introduction—The Biology and Pathology of Squamous Cell Carcinomata in the Head and Neck

Newell W. Johnson

Abstract A very wide range of homeostatic processes are disturbed in cancer. These are visible at patient, lesion, tissue and cellular levels and are increasingly understood at a molecular level, providing many opportunities to research molecular therapeutic targets. This chapter introduces and contextualises most of the processes currently understood as of importance to the biology of cancer: many of these understandings are comparatively new; some are speculative. It is upon such speculations and the application of scientific method that progress in cancer prevention and management depend.

1.1 Introduction

In an era of vast expansion in knowledge of cell and molecular biology, and in many technologies which enable vast datasets to be generated, it is wise to go back to basic biology: to emphasise that molecules behave only within cells; that cells interact with their neighbours of both similar type/function and with surrounding supporting and infiltrating cells; that these behave within tissues; tissues comprise organs; and the whole "maketh the man". Further, individual human beings exist within family and community structures, the behaviour of which influences risks and resistance to disease, including cancer. There are dangers in studying molecules, cells and people in isolation.

This chapter attempts to take us back to the basics of mammalian biology with, necessarily brief, explanations of the mechanisms involved—of homeostasis—and of how these are deranged in cancer. Examples are drawn from one major type of neoplasm—squamous cell carcinomata (SCC)—as these are the major public health

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© Springer Science+Business Media B.V. 2017

S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_1

challenge amongst head and neck cancers (HNSCC). Many of these biological processes are explained in detail in subsequent chapters where research has reached a sufficient stage for the control pathways to be targeted in patient care, especially so in the current era of personalised cancer care.

Many modern textbooks cover these topics in greater detail than is possible here. Bernier (2011) has specific chapters dealing with most aspects of cancer biology with a focus on head and neck oncology. Other more general, and very detailed, resources are: Weinberg (2013), Mendelsohn et al. (2015) and Pelengaris and Khan (2013). Rather than summarise, or worse plagiarise, these texts, the approach taken in the present chapter has been to use information from international peer-reviewed journals, and understandings, prejudices and questions derived from this author's experience of head and neck oncological research and patient care.

1.2 Genetic and Epigenetic Drivers and Controllers

Current dogma has it that cancer is a genetic disease, by which is meant that an irreversible change in a gene or genes within somatic cells, or in its/their control, has created and will maintain growth of both the primary, and any secondary neoplasms. Genes can by altered by duplications or deletions in whole or parts of chromosomes, by mutations in individual genes, by silencing of normal gene expression by epigenetic events in the environment and by aberrant signalling which up-, or down-regulate gene expression.

For several decades in the latter part of the last century dogma had it that $\sim$6 mutations in key genes was both necessary and sufficient to cause cancer. This has always seemed unlikely to the present author: which six?; how much can the cluster differ by type of cancer/tissue of origin?; how variable is the cluster by aetiology?; how variable will the pattern be within and between individuals?; given the additional mutations which comprise what we call "tumour progression" and the outgrowth of new clones, which of these are significant or just noise? Added to all of this, we are currently learning a good deal about the epigenetics of cancer, a process whereby during embryogenesis and throughout life, methylation of DNA or modification of histone proteins can silence particular genes (Fig. 1.1).

Studies of individual patients, of case-series and Genome Wide Association Studies (GWAS) of large populations produce long lists of affected genes, their frequencies in different types of cancer and racial/ethnic groups, but the associations remain statistical concepts: there may well be some common final pathways in malignant transformation and in many subsequent behaviours, but the genesis of a neoplasm may be specific to—even unique—for every patient. *We should regard every neoplasm as an unique biological event in an unique host*! Each patient's metabolism and immune response will be different. The current fashion for personalised care properly takes cognisance of this.

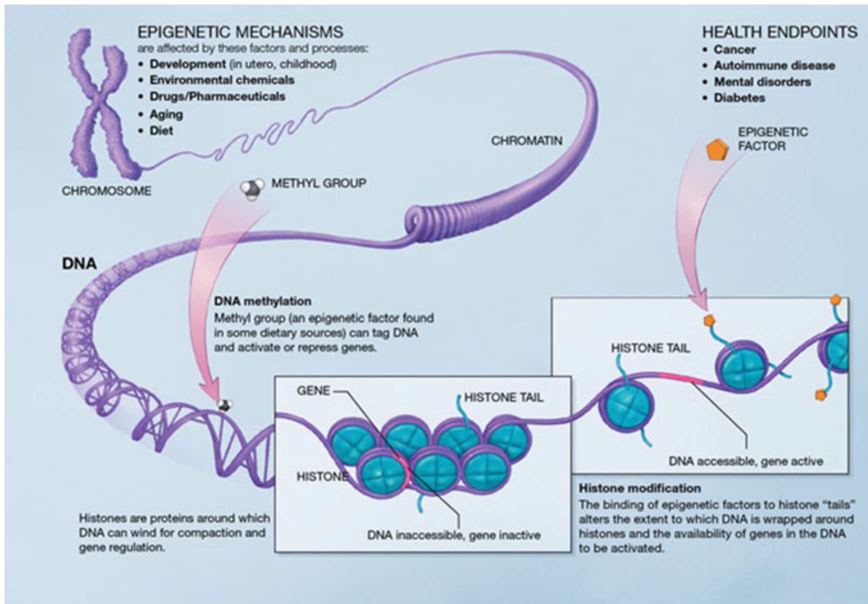

Fig. 1.1 Epigenetic mechanisms, DNA methylation and cancer. With permission from the "National Institutes of Health". http://commonfund.nih.gov/epigenomics/figure.aspx

A huge effort is being made sequencing all of the common, and not-so-common cancers. Recent data release from the International Cancer Genome Project (https://icgc.org/), (http://www.genome.gov/17516564) and, the Cancer Genome Atlas in the USA and several Collaborators including the Wellcome Trust Sanger Institute (https://www.sanger.ac.uk/research/projects/cancergenome/) describes 50 collaborative projects with neoplasms taken from 18 primary cancer sites in 12,232 donors, revealing 9,871,477 simple somatic mutations in a total of 57,526 mutated genes. How many of the genetic changes in malignancy are effect rather than cause? Structural and functional changes in genes increase in number and type with the phenomenon of "tumour progression" (vide infra): every malignant cell can contain a different set of genetic aberrations; most might be regarded as epiphenomena; many are not compatible with cell viability let alone cell division; functions which are fundamental to the continued presence of stemness? Such epiphenomena may be important in key aspects of behaviour, including increase in the mass of a "tumour"; cell mobility and infiltration; propensity to metastasis, abnormal secretion, e.g., but whilst "correction" of such a malfunction may have clinical benefit, cure remains impossible whilst the host—the patient—lives unless every cell with the ability to be itself immortal is killed or permanently suppressed.

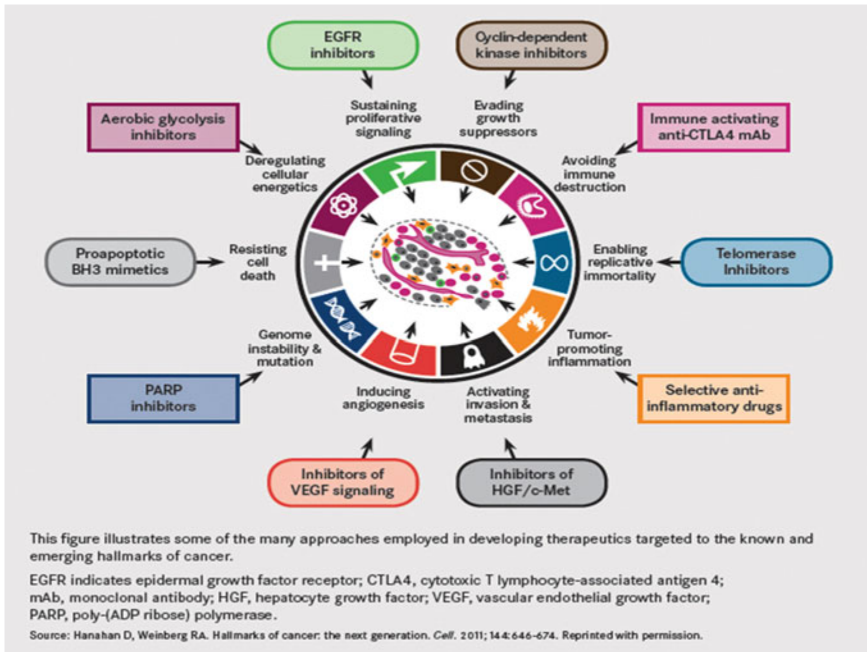

Fig. 1.2 Hallmarks of cancer and therapeutic targets (reproduced with permission from Cell 144, March 4, 2011; p. 647)

1.3 The Hallmarks of Cancer

Figure 1.2 illustrates 10 functions of a tissue essential for it to behave as a malignant neoplasm: here some drugs which interfere with these processes, the focus of this Volume, are listed also. How many of these hallmarks are necessary or sufficient for progression of the disease in any particular patient?

1.3.1 Accelerators, Brakes and Maintenance Men

There are a bewilderingly large number of cell signalling pathways: cell surface receptors binding messenger molecules which transmit signals via a large number of extremely complex pathways to the nucleus, where gene functions are either up- or down-regulated. These are understood to variable degrees, but sufficiently in many cases for their dynamics to be used in diagnosis, prognosis and treatment with drugs and antibody inhibitors. Reproduced here with permission from “Cell” (Fig. 1.3), simply as indicator of the complexity, and raising the question “How many, and which, specifically, of these many genes need to be mutated for malignant transformation of a cell”?

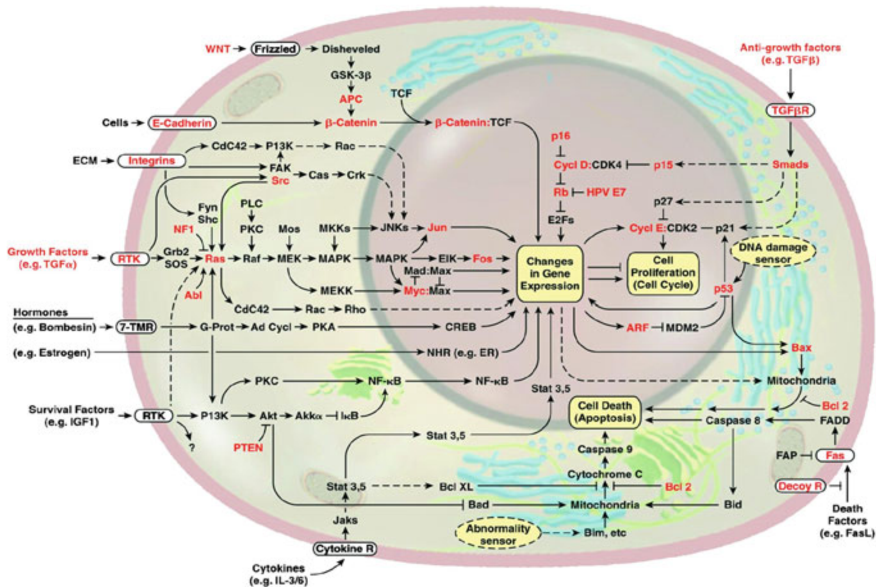

Fig. 1.3 The circulatory of cancer genomics. Reproduced with permission from Hanahan and Weinberg, published in Cell (2000)

An attractive scenario was prevalent in conversations a decade or so ago and is still found on public websites. http://www.cancer.org/cancer/cancercauses/geneticsandcancer/genesandcancer/genes-and-cancer-oncogenes-tumor-suppressor-genes. This had it that as few as 6 mutations were critical, and sufficient if one or more of these disturbed the process of cell division (by analogy the accelerator of a vehicle); one or more the brakes (control of apoptosis or other processes of cell death); and one or more the ability for DNA repair (maintenance of the vehicle): the vehicle would crash of the accelerator was stuck on; the brakes failed, or the wheels fell off.

Recently, merging the expertise of mathematical modelling with that of cell and molecular biology, it has been proposed that in most epithelial neoplasms, three mutations may be sufficient (Tomasetti et al. 2015). Neoplastic progression (vide infra) will introduce many more, so that it remains a major challenge to determine which type of aberration in which gene might be altering a pathway the correction of which can improve the outcome for a given patient.

1.4 Epithelial Cell Origins: Embryology

All squamous cell carcinomas (SCC) are presumed to arise from a cell or cells of mature adult stratified squamous epithelia, viz: from the epithelial lining of the mucous membrane of the upper aero-digestive tract (UADT) (Fig. 1.4).

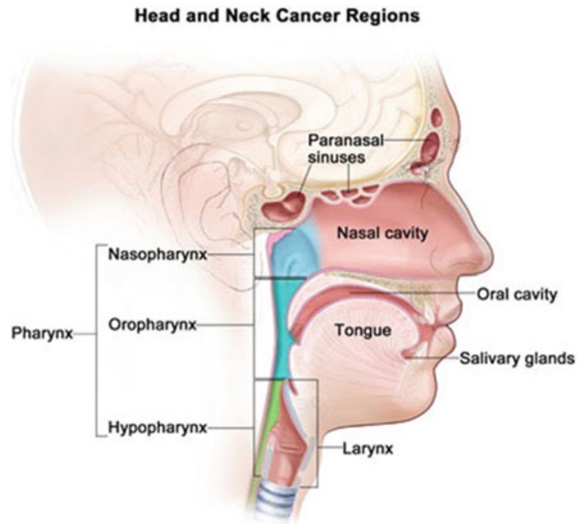

Fig. 1.4 Head and neck cancer regions

Transformed keratinocytes constitute the *neoplastic population* and these cells, together with supporting stroma and reactive, infiltrating immune/inflammatory cells together comprise a new tissue which is the *neoplasm* itself (from Ancient Greek νεο- neo- "new" and πλάσμα plasma "formation, creation"). The term "tumour/tumor", although in common use, is deprecated because this simply means a swelling (from the Latin, tumeō "I bulge, swell"), and such swellings can arise from inflammation, oedema, obstruction of a viscus and several other causes. Equally, derived terms such as "tumorigenesis" are inappropriate although, unfortunately, they are frequently used.

Malignant neoplasms, especially those described as well-differentiated, thus resemble the parent tissues, the epithelial component showing morphological transitions from basal cell types, through a stratum spinosum, and layers of cells containing increasing amounts of keratin which, as part of this maturation or differentiation process, lose their nuclei and flatten. The keratinocyte population which continues to proliferate, expands inwards as well as outwards, this being the process of infiltration. As keratinocyte progeny leave the basement membrane they mature towards the centre of promontories (appearing as islands in a thin slice or histological section) forming the keratin pearls typical of well-differentiated SCC. The mature cells, shed from the surface of healthy skin or mucous membranes, have "nowhere to go", so add to the bulk of the neoplasm forming, in the literal sense, a tumour (Fig. 1.5).

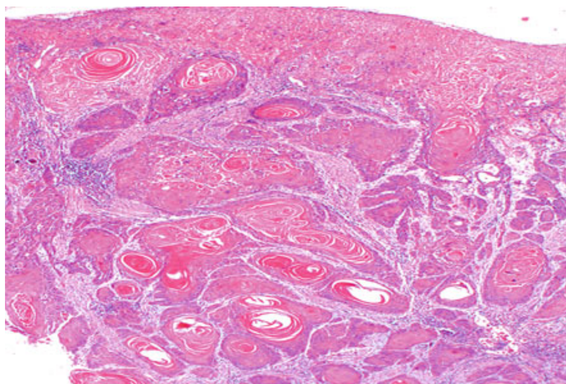

Fig. 1.5 A typical well-differentiated SCC of the oral mucosa with marked formation of keratin pearls. The body surface is above the tumour in this image. There is comparatively little stroma but note, even at this low magnification, the infiltration of inflammatory cells

1.4.1 Embryological Origins of HNSCC

Whether HNSCC arises from ectoderm or endoderm, viz: from a mucosal site anterior or posterior to the bucco-pharyngeal membrane, appears to be of little consequence in terms of the intrinsic behaviour of transformed keratinocytes. Rather it is anatomical factors, principally surgical access and the complexity of lymphatic drainage, which make cancers of the tongue more dangerous than those of the cheek, or (with the exception of HPV-related neoplasms) the pharynx more dangerous than the lip or mouth.

1.5 Epithelial Cell Origins: Stem Cells in Head and Neck SCC

The Chapter by Gonzalez-Moles in the present Volume provides a comprehensive review of this topic. Briefer coverage here places the concepts into perspective. In health, the human genome continues in its entirety from a fertilised ovum through multiple divisions and steps in differentiation and specialisation to form adult tissues of all types. It is the progressive shutting down of blocks of genes, whereby repressor proteins attach to silencer regions of particular genes, and the switching on or up-regulation of others, which lead to differentiation of tissue type. Once distinct tissues develop—and function, environmental messages can influence gene expression, and these may be heritable. Any healthy adult cell can theoretically recreate a whole tissue type, even a whole animal, given the right growth factors and environment. In practice embryonic stem cells, or those that remain multi-potent in the adult, such as can be derived from bone marrow or dental pulp, are easier to engineer. There is a vast market nowadays in stem cell treatments for tissue regeneration in many diseases, e.g. following stroke, macular degeneration. The field is advancing rapidly and seminal papers appear in many issues of the world's leading

scientific journals, e.g., at the time of writing, a series of outstanding papers in the December 2014 issue of Nature (Tonge et al. 2014). The progress of this work can be tracked through the user-friendly website of the international consortium “Project Grandiose” whose portal is at http://www.stemformatics.org.

The concept of cancer stem cells is, however, somewhat different. It has long been known that bone marrow contained a population of stem cells which, through a series of differentiation steps, gives rise to erythrocytes, lymphocytes or granulocytes. In epithelia, a subpopulation of cells have equivalent behaviour: This is a situation where a minority of cells, when they divide, duplicate their DNA template for a single daughter cell which moves away and the parent cell retains its anatomical position. The daughter cell then divides producing two third generation cells and each of these produce an expansion of the required cell numbers, for a genetically pre-determined number of divisions. There is a genetically predetermined switch to keratinocyte differentiation. From the pioneering work of Mackenzie and of Potten we know that, in skin, a single epithelial stem cell serves an anatomically defined EPU (Epithelial Proliferation Unit) (Mackenzie 1970; Mackenzie et al. 1981; Potten 1974). Similarly, the microvilli of the gut have a distinct organisation into compartments; in mucosal epithelia (Humphries and Wright 2008); these however, have proved harder to visualise.

Thus the *rate* of division of *stem cells* and of *expansion compartment* cells is critical in homeostasis. Equally critical is the *accuracy* of the process of DNA replication. Random errors can produce immortalisation in a clone of progeny. If cells still in the stem cell compartment or in the viable expansion compartment undergo a mutation or other alteration in one—more likely several—of the genes listed in Table 1.1, above, a malignant neoplasm can result. Theoretically this could be either from a failure of the genetic control of the switch to differentiation pathway, and/or a failure of control of the rate of division of stem cells. There are no reliable methods for making such a distinction but a very recent paper by Tomasetti and Vogelstein (2015) has attracted much interest in this regard. These authors argue that the risk of a malignancy in a particular tissue is proportional to

Table 1.1 Genes which are typically altered in HNSCC, and the functions affected

Tumour suppressors	*p53*, *Rb*, *MTSI*, *RARβ*, *p21*, *DOC-1R*
Oncogenes	*MDM2*, *MYC*, *RAS*, *EGFR*, *FOS*
Cyclins	*CYCLIN D1*
Apoptosis	*BCL-2*, *BAX*, *APOVFAS*, *TELOMERASE*
Cancer susceptibility	*GST-M1*, *CYP1A1*, *MTSI*
DNA instability (MS1)	*3p*, *4q*, *5q*, *6p*, *7q*, *7p*, *9p*, *9q*, *11q*, *13q*, *14q*, *18q*, *17p*
Mismatch repair	*hMLH-1*, *hMSH-2*
Angiogenic factors	*VEGF*, *FGF*, *ENDOTHELIN*
Heat shock proteins	*HSP70*, *HSP47*, *HSP27*
Proliferative markers	*Ki67*, *PCNA*, *MYB1*
Invasion metastasis	*ETS1*, *MMPs: ST2*, *ST-3*, *COLLAGENASE*, *uPA*
Drug resistance genes	*p-GLYCOPROTEIN*, *GST-pi*

the number of (stem) cell divisions in that tissue required throughout life for homeostasis: a stochastic model in which the chance of a random mutation or mutations being sufficient for neoplastic transformation increases with the number of times DNA has been replicated. The argument relates to stem cells, but could equally apply to expanding cell compartments in epithelial tissues. They test this concept by plotting the rate of (stem) cell division in human tissues against incidence of cancer in that tissue or organ and show a strong positive correlation of ~ 0.81. They argue that about two thirds of cancers arise simply due to "bad luck"—due to such random errors, the remaining third being explained by environmental carcinogens. This is proffered as an explanation for the low incidence of malignancy in tissues such as brain, and for sarcomata in general compared to epithelial cancers, or for small bowel v colon. The epidemiological data come from the SEER database in the USA, so more work will be necessary to see if the theory is dented by the large differences around the globe in many cancers, notably tobacco-associated SCC.

This thinking has profound impact on public health policy making. Although it will vary from population to population, depending on the prevalence of established risk factors therein, primary prevention will have limited impact. For random cancers to be managed on a population basis, increased secondary prevention—screening for early detection of smaller, perhaps asymptomatic, more curable, lesions is needed.

The Cairns hypothesis, promulgated in a classic paper in 1975, and revisited with mathematical modelling in 2002, proposes that stem cells retain their original DNA template throughout life (Cairns 1975, 2002). In this case if a mutation arose during replication of a stem cell, this would be present only in a member of the expansion compartment, and would automatically die out. This was postulated as a mechanism for protection against cancer or other genetic diseases. Conversely, however, if a stem cell carries an oncogenic mutation, a neoplasm is inevitable. Successful treatment would necessitate elimination of affected stem cells and this has become something of a dogma in contemporary cancer research.

The phenomenon of "tumour progression" (see below) is more consistent with "stemness" being a fluid or changeable property of sub-populations of cells within a solid neoplasm, as in the stochastic model proposed by Antoniou et al. (2013) (Fig. 1.6).

1.5.1 Stem Cell Markers in Epithelial Cancers

Most of the literature, and the better experiments and models derived therefrom, comes from liquid neoplasms, i.e. haematological cancers (Nguyen et al. 2012; Antoniou et al. 2013). Much effort has gone into defining the wider characteristics of, and defining markers for, stem cells in solid cancers in recent years, and panels are available from several commercial companies. As can be seen from Table 1.2, the functions ascribed are diverse and non-specific. Such markers are widely used in

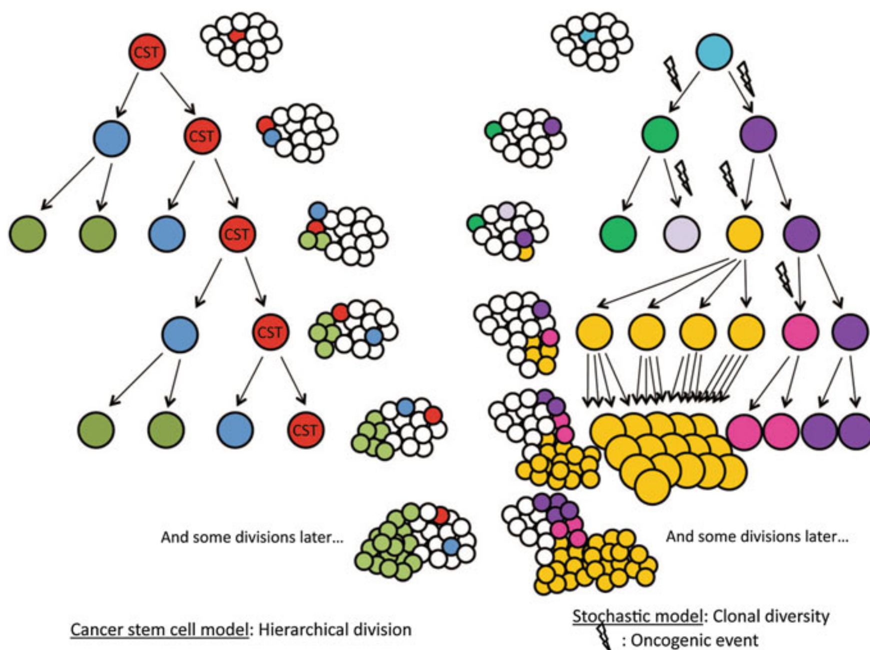

Fig. 1.6 The stochastic model of cancer stem cells compared with the "traditional" model. From Antoniou et al. (2013). Reprinted by permission of Taylor & Francis, LLC

Table 1.2 Solid "tumour" cancer stem cell markers

• **ABCG5**: ATP binding cassette family member; transport of sterol and other lipids. Confers doxorubicin resistance
• **CD90 (Thy1)**: Signal transduction, possible role in stem cell differentiation
• **ALDH1**: Aldehyde dehydrogenase (ALDH) family. Role in the conversion of retinol to retinoic acid, an important pathway for proliferation, differentiation and survival
• **CD133 (Prominin1)**: Transmembrane protein. Expressed on developing epithelia
• **CD24 (HSA)**: Co-stimulatory role in B and T cells. Only known ligand for P-selectin. Low levels can characterize breast tumour-initiating cells. Potential cancer stem cells reported to be CD24+/CD44+
• **EpCAM (epithelial cell adhesion molecule, ESA, TROP1)**: Homophilic Calcium ion-independent cell adhesion molecule
• **CD44 (PGP1)**: Multiple isoform adhesion molecule, roles in signaling, migration, and homing. CD44H, high affinity for hyaluronate; CD44V confers metastatic potential
• **Hoechst SP (side population)**: Hoechst 33342 efflux pump present on the plasma membrane of many cell types. Activity conferred by the ABC transporter ABCG2. Inhibited by verapamil

Adapted from http://www.promab.com/services/cancer-stem-cell/identification-platform

research using cell lines, including HNSCC described earlier by us and others (Qiao et al. 2011; Shah et al. 2014), so we must remind ourselves how artificial such systems are and not over-interpret such studies. Several of these markers e.g. CD 40, CD 93, CD 140 and aldehyde dehydrogenase (ADH) have also been used in immunohistological staining to demonstrate "cancer stem cells" in hepatocellular carcinomas (Lingala et al. 2010).

1.6 Epithelial Cell Proliferation: Circadian Rhythms and Derangements in Cancer

All epithelial tissues continue to proliferate throughout life—indeed even for a time after death. This is an exquisitely controlled process, designed so that the adding of new cells is precisely balanced with the need for developing the tissue or organ in growth and development phases of life (from embryogenesis to adulthood); with the loss of mature cells through desquamation and apoptosis during adulthood to keep structures in a healthy mature state (homeostasis). As we age there is an inevitable imbalance, with atrophy and some loss of function. But if you don't use it you lose it, and epithelia remain able to respond if functionally stimulated at any age. This applies to all tissues and organs, including the central nervous system, as is increasingly recognised: this applies to all epithelia, including solid organs like liver and pancreas, which retain renewal and regenerative capacity.

Here we are concerned with stratified squamous epithelia. Cell division in basal layers, cell movement, maturation and shedding are under precise control, with a recognised diurnal variation (Warnakulasuriya and MacDonald 1993) and with chemical signals from cells moving towards the surface instructing cells in the proliferation compartment to divide, depending on their position in the passage to the surface, and the thickness of the epithelium required. Abrasion or erosion triggers increased signal. This is best understood by the theory of control by chalones: leakage of these chemical messages stimulates cell division by a negative feedback loop. Chalones were first described by Bullough and Laurence (1968) in the 1960s and Elgjo and colleagues (2004), after a flurry of research activity in the 1970s, have returned to contention: the properties are now thought to be those of member(s) of the family of Growth Factors, the TGFβ Superfamily (Elgjo and Reichelt 2004) which act through the Smad pathway.

Stimulatory factors include, in addition to TGFs: human epidermal growth factors (EGF); Interleukins -1 and -2 (IL-1, IL-2, IL-6); GM-CSF (colony stimulating factor); Basic FGF (fibroblast growth factor); keratinocyte growth factors (KGF); Vitamin A and associated retinoids; androgens. These all act by binding with specific receptors on the cell surface, amongst the most important of which are the EGFR/ERBb family. Drugs based on monoclonal antibodies designed to block EGFR, are now licenced for the treatment of HNSCC, the only true biotherapeutic currently licensed for treatment of HNSCC. Such approaches are the basis of

personalised medicine: only HNSCC patients whose neoplasm over-expresses EGFR, and only those breast cancer patients whose neoplasm overexpresses HER2 receptors, will benefit from Cetuximab and Herceptin treatment, respectively. The current situation is described on detail in Chap. 6 by Schmitz and Machiels in this Volume.

Physiological inhibitors of keratinocyte proliferation include IFNα, IFNγ, TNFα, adrenaline, glucocorticoids and high local calcium levels.

The multiple pathways of cell proliferation are reviewed by Feitelson et al. (2015) with particular emphasis on natural compounds which cause interference and have value as anticancer agents.

1.7 Epithelial-Mesenchymal Transition (EMT)

The process of EMT is intimately related to concepts of cancer stem cells, and is explained well in a valuable paper by Biddle and Mackenzie (2012). See Fig. 1.7 reproduced there from.

EMT and the reciprocal mesenchymal to epithelial transition (MET) are key processes involved in both tumor metastasis and stem cell differentiation and

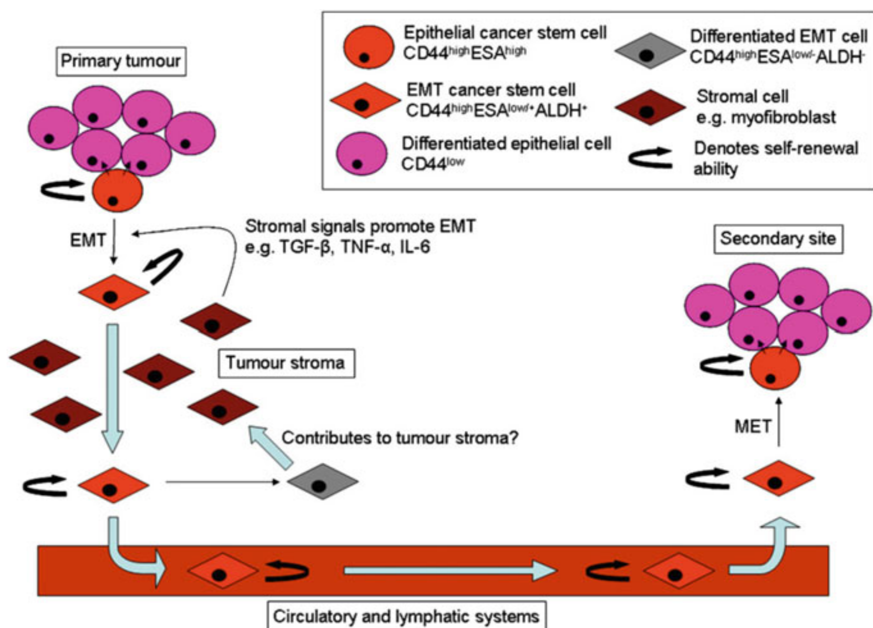

Fig. 1.7 A schematic diagram illustrating epithelial-mesenchymal interaction and reciprocal mesenchymal to epithelial interaction (republished from Biddle and Mackenzie Cancer and Metastasis Reviews, June (2012) with permission from Springer)

development. During EMT, epithelial cells lose their apical and basolateral polarity, break their intercellular tight junctions, and degrade basement membrane and extracellular matrix components to become migratory mesenchymal cells.

Defining markers of EMT, and of the cell surface changes and intracellular pathways involved, is part of current cancer research dogma. Using real-time PCR, key genes that either change their expression during this process or regulate those gene expression changes can be identified. A PCR Array that profiles 84 key genes has been developed and marketed by sabiosciences.com http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-.

Many of these changes are triggered by members of the TGFβ/BMP superfamily, are mediated via snail and slug gene expression, and involve increases in matric metalloproteinases (MMPs), as we, and others, have shown with HNSCC cell lines (Qiao et al. 2010; Masui et al. 2014). More than one phenotype of EMT in HNSCC cell lines can be recognised, e.g. that mediated through the Glycogen synthase kinase 3β pathway (Shigeishi et al. 2013; Schramm 2014). Many of the characteristics are those of myeloid cells.

Most of these functional, or behavioural, changes are reversible, as determined by studies in cell culture (Larue and Bellacosa 2005). There is also the overlap with markers of epithelial stem cells. Taken together, this reversibility speaks against both the concept stemness and against malignant transformation being a unique or catastrophic event in a tissue.

1.8 Energy Metabolism and Mitochondrial Function in Neoplasms

For a neoplasm to survive and expand, it is self-evident that it has to be metabolically efficient. This applies to the neoplastic population itself and to the development and continued supportive function of the stroma. If it were possible to “tie off” the blood supply, the neoplasm would infarct but this is not possible with malignant lesions because of the widespread and diffuse recruitment of new blood vessels.

At a cellular and molecular level research has addressed changes in the metabolism of the neoplastic cells themselves: mutations and dysregulation of energy pathways which give a growth advantage. It is fundamental to all tissues that the ATP (adenosine triphosphate) required for a cell’s energy comes from the normal process of oxidative phosphorylation in mitochondria, and from glycolysis in the cytoplasm at times of lowered oxygen availability. We have shown, both in experimental carcinogenesis in an animal model, and in human OPMD, increased glucose-6-dehydrogenase activity in dysplastic keratinocytes (Evans et al. 1980). These data were indicative of an increased use of the pentose phosphate shunt—which provides a major source of NADPH and of ribose for synthesis of DNA and RNA. This might indicate risk for neoplastic transformation, although a single metabolic marker cannot alone have high positive predictive value, and because it is a normal function, will be non-specific.

The famous Warburg effect, postulated in 1930, sates that neoplastic cells preferentially utilise the anaerobic pathway, with production of lactate, even in the presence of adequate oxygen (Warburg 1956, 1969). Interestingly, it is this enhanced glycolysis which enables PET (positron emission tomography) to identify a possible malignancy because of increased glucose uptake. Whether this switch is a primary cause, or an effect, of neoplasia remains controversial but with our enhanced knowledge of the controls operated by oncogenes and tumour suppressor genes, this field of research is undergoing a resurgence and is beautifully reviewed by the Frezza group at the University of Cambridge (Gaude and Frezza 2014; Sciacovelli et al. 2014) (see Fig. 1.8). For example the metabolic switch is favoured by the activity of oncogenes such as c-Myc and by mutations in a number of genes controlling mitochondrial enzymes.

Their legend reads: Mitochondrial dysfunctions in cancer. Schematic representation of mitochondrial enzymes involved in cancer, focusing on enzymes of the TCA cycle (A) and of the respiratory chain and ATP synthase (B). The type of cancer associated with each individual enzyme is listed in boxes. The color of the text indicates if the enzyme has been found upregulated (red), downregulated (blue), or mutated (black) in the given tumor type. CS citrate synthase, Acoaconitase, IDH isocitrate dehydrogenase, IDH* mutant IDH, OGDH oxoglutarate dehydrogenase, SDH succinate dehydrogenase, FH fumarate hydratase, ME malic enzyme, MDH malate dehydrogenase, PDH pyruvate dehydrogenase, OG 2-oxoglutarate, 2HG 2-hydroxyglutarate, HLRCC hereditary leiomyomatosis and renal cell cancer, PGL/PCC hereditary paraganglioma and pheochromocytoma, CI–CV complex I–V, Cyt c cytochrome c, UQ ubiquinone, UQH2 ubiquinol, ROS reactive oxygen species, ATPIF ATP synthase inhibitory factor. Dashed lines indicate a series of reaction in a complex pathway, whereas solid lines indicate a single step reaction.

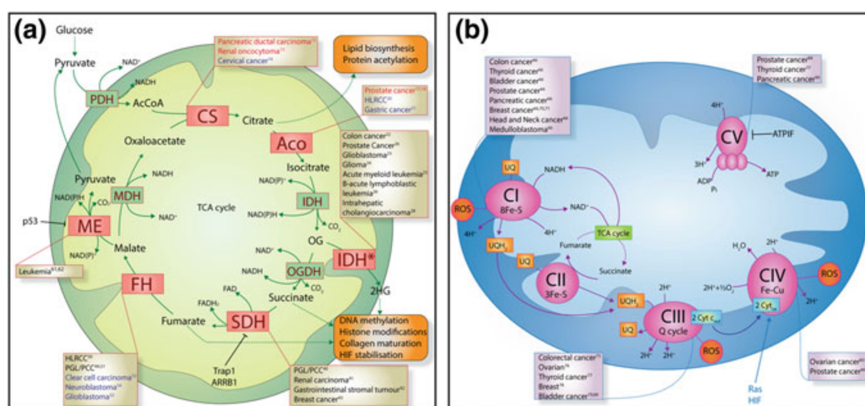

Fig. 1.8 Mitochondrial enzymes involved in cancer. Reproduced from Gaude and Frezza (2014) (reproduced with permission by the authors)

Fig. 1.9 Schematic illustration of cancer progression in man (courtesy N.W. Johnson)

In contrast, Seyfried and colleagues argue that cancer is fundamentally a metabolic disease arising from defects in the structure and function of mitochondria. This forces glycolytic metabolism in transformed neoplastic cells and raises the possibility of treatment by training the patient to adapt to a ketone generating diet; restrict carbohydrate and substitute with fats. Normal cells can adapt, cells of the neoplasm cannot: the effect is anti-angiogenic, anti-inflammatory and pro-apoptotic (Sayfried et al. 2014).

Drugs which target upstream metabolic pathways are an increasing focus of research (Kim 2015).

1.9 Tumour Progression in Head and Neck SCC

In the evolution of the many clones which emerge as a neoplasm grows, Boshoff suggests that H&N SCC evolve more like a bush than a palm tree: viz. the branching happens early with much consequent complexity (Boshoff 2013) (Fig. 1.9).

1.10 Epithelial Cell Cohesion and Movement

All normal epithelial tissues have close cell to cell contact, with a minimum of intercellular material. They adhere to each other, and to the basement membrane, using specific adhesion molecules. These are listed below (from Thomas and Speight 2001) (Table 1.3).

These cell adhesion molecules (CAMs) have complex transmembrane structures, with an extracellular, surface, binding site and cytoplasmic domains from which signals pass through cascades to influence gene regulation. Defects in these attachment mechanisms can promote carcinogenesis (Fig. 1.10).

Table 1.3 Oral epithelial adhesion molecules—listing the classes of adhesion molecules expressed by keratinocytes in normal oral epithelium together with their ligands and functions

Family	Expression by oral epithelium	Liqand	Functions
Integrins	α2β1	Collagen, laminin	Principal cell-ECM receptors. Shown to be involved in many keratinocyte functions, including migration, differentiation, protease production, basement membrane assembly
	α3β1	Laminin	
	α5β1	Fibronectin	
	α9β1	Tenascin	
	α6β4 (component of hemidesmosomes)	Fibronectin	
	αvβ5	Vitronectin	αvβ6 expression is associated with healing
	αvβ6	Fibronectin	
Cadherins	E-cadherin	Homotypic, also αE β7	Primarily involved in homotypic reactions maintaining cell-cell contact and tissue architecture. E-cadherin forms adherens junctions between cells; desmosomal cadherins form dsmosomes
		Integrin on lymphocytes	
	P-cadherin (basal cells only)	Homotypic	
	Desmogleins 1-3	Homotypic	
	Desmocollins 1-3	Homotypic	
Selectins	E-selectin reported on Inflamed gingiva Selectin ligand SLex on Basal keratinocytes	Sialylated and fucosylated Carbohydrates such as sLex, sLea	Role uncertain in keratinocytes. Possibly involved in immune response
IgSF	ICAM-1 on junctional Epithelium ICAM-1 expression in inflammation	β2 integrins on neutrophils, monocytes, lymphocytes	Immune response. Increases inflammatory cell migration and attachment in conditions such as lichen planus
CD44	CD44s Multiple splice variants, including v2-10, v3-10, v4-10, v6-10 and v8-10	Principally hyaluronan, can also bind growth factors and other matrix molecules	Role uncertain in keratinocytes. Splice variants possibly involved in migration and proliferation

1.10.1 *Epithelial Cell Movement and Derangements in Cancer*

Keratinocytes are constantly on the move. Movement from the basal layers to the surface, and desquamation, involves several active processes. These utilise well-known contractile proteins in the cytoplasm: especially actin; the process involves programmed unlinking and relinking of cell attachments, particularly desmosomes. A high degree of motility is a fundamental property of all malignant cells: increased expression of actin has long been investigated as a marker of the malignant potential of "premalignant" tissues, as has the loss or down-regulation of cell attachment molecules, particularly ICAM.

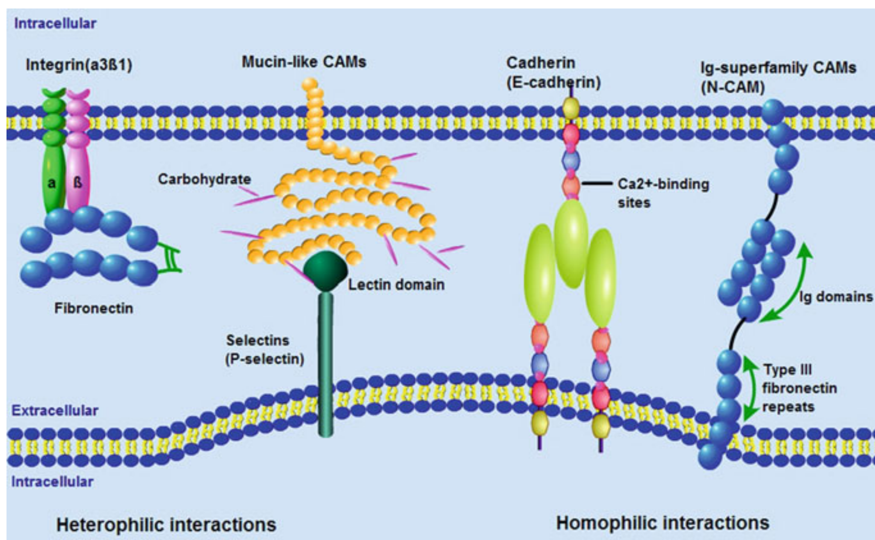

Fig. 1.10 A schematic diagram illustrating cell adhesion molecules (CAMs) with an extracellular surface molecule, binding site and cytoplasmic domains (reproduced from SAB Signalway Web site, with permission) http://www.sabbiotech.com/images/upload/Image/adhersion.jpg

Epithelial-mesenchymal transition (EMT) is a phenomenon which has attracted much recent interest. This is a situation where cells assume a morphology more akin to mesenchymal cells, in that they become spindle-shaped and can be shown to migrate in cell-sheet cultures, or form spheroids in organotypic cultures (vide infra).

Historic views of cell determinism, viz: that once the germ cell layers are formed in the embryo, epithelial and mesenchymal cells and their progeny are forever committed to that lineage, are no longer held. (it is well established now that the nucleus of a skin keratinocyte, which contains a whole genome, can reproduce a whole animal, if it is located in a permissive cytoplasm which enables appropriate genes to be switched on or off). Nevertheless, because the defining characteristic of epithelia is their propensity to “stick together” as a tissue, the concept of EMT has been found attractive by many.

In SCC, epithelial markers remain key to diagnosis. Most such carcinomas in the H&N retain sufficient morphological characteristics of the parent tissue to make histomorphological classification straightforward. However, the well-recognised but comparatively rare spindle cell variant of H&N SCC can be a diagnostic challenge unless immunocytochemical identification of epithelial characteristics is utilised: these would be predominantly using monoclonal antibodies to identify cytokeratins.

Movement of keratinocytes in wound healing and in cancer invasion requires a signal: one such is the water soluble lipid, lysophosphatidic acid, derived from the circulation. Note the importance again of the TGF family pathways.

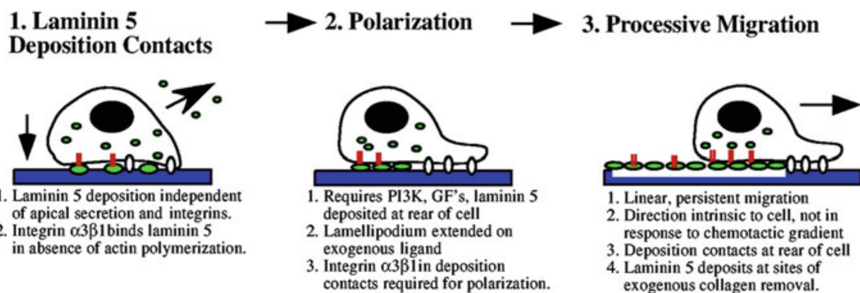

Fig. 1.11 Role of Laminin 5 deposits in cell migration (With kind permission from the author Dr Diane Frank: J Cell Sci 2004)

Migration also requires a substrate, frequently provided by laminins, when metalloproteinases from the keratinocyte have degraded collagen (Fig. 1.11).

1.11 Epithelial Cell Death: Telomerase and Apoptosis; Non-apoptotic Cell Death

Given that epithelial organs and tissues are constantly proliferating and renewing themselves, in both health and disease, it is clear that cells have to be disposed of as part of homeostasis to maintain structure and function. In skin and mucous membranes this occurs primarily by surface desquamation, the internalised surfaces of an invasive cancer accumulating the products. Apoptosis—programmed cell death—is another essential method of control. Anoikis is a recently coined term for the promotion of apoptosis in cells detached from each other and/or from their normal matrix binding mechanisms. Nucleated keratinocytes have "Death Receptors" on their surface, these being, again, members of the TNF superfamily. In neoplasms such signalling can be blocked by mutations or by viruses.

There is evidence to suggest that expansion of a population of malignant keratinocytes may depend more on reduced or deficient apoptosis, than on the rate of cell proliferation (Naresh et al. 2001). Thus pathways of apoptosis are the subject of much current research, in particular the mTOR pathway, discussed in detail in Chap. 7 in this Volume by Gutkind and co-authors. Survivin, a protein product of the BIRC5 gene, a key inhibitor of apoptosis by inhibiting caspase activation, is discussed in detail in Chap. 8 by Khan.

Apoptosis has to be distinguished from *necrosis*, in which cell death occurs as a result of damage, infection or loss of nutrients. *Regulated* necrosis, on the other hand, describes a number of newly discovered pathways with potential for therapeutic interventions (Vanden Berghe et al. 2014).

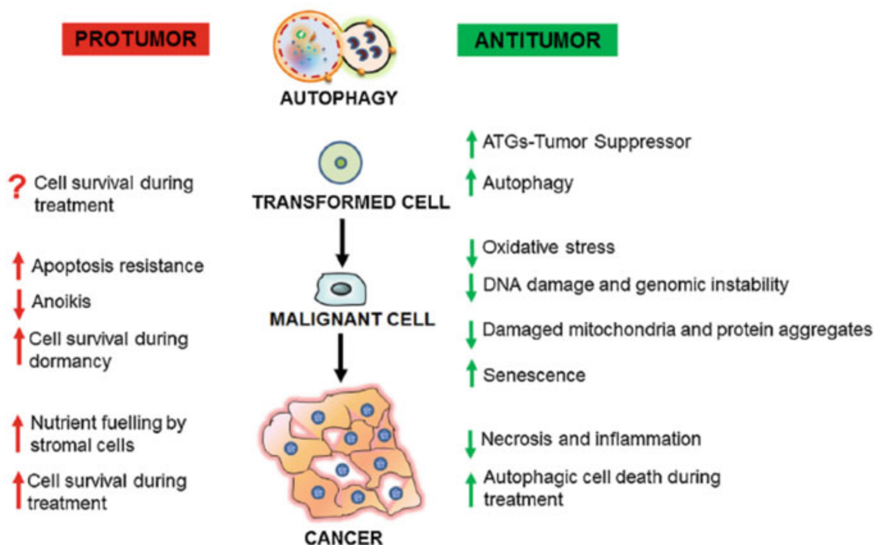

Fig. 1.12 Pro-survival and pro-death functions of autophagy (reproduced with permission from Elsevier: Seminars in Cell and Development Biology 2015)

Autophagy or autophagocytosis is the normal homeostatic process whereby damaged or defunct cytoplasmic components fuse with lysosomes and are degraded in autophagosomes (Fig. 1.12).

Autophagy has been implicated in many different diseases, including neurodegeneration, cardiac myopathy, autoimmune disease and cancer. The role of autophagy in cancer is complex and paradoxical. While autophagic deficiency has been shown to promote carcinogenesis in animal models, autophagy may actually support tumor growth by enhancing cancer cell survival in the face of nutrient depletion or accumulation of toxic molecules. GeneTex has introduced antibodies for autophagy research.

http://www.genetex.com/Web/News/NewsList.aspx?id=362, accessed 03 June 2015.

Autophagy is regarded as a fundamentally protective mechanism for healthy cells. However autophagy can contribute to survival of damaged or starved malignant cells, so that inhibition of autophagy can be used in cancer therapy. The antimalarial drug chloroquine, which enhances p53 function and inhibits the later stages of autophagy, can enhance the effects of some anticancer drugs and, perhaps, radiotherapy (Zhang et al. 2015). There is a huge current literature across many types of neoplasms. The ying and yang of autophagy, and the molecular pathways involved, are thoroughly reviewed by Galluzzi et al. (2015) and by Panda et al. (2015) (Fig. 1.12).

Part of the oncogenesis of Epstein-Barr virus (EBV) may be via inhibition of autophagy (Fotheringham and Raab-Traub 2015). A comprehensive review of the various mechanisms of programmed cell death and of potential ways of manipulating these in cancer therapy has been presented recently by Fuchs and Steller (2015). The relative importance of the several pathways is different at different stages of initiation, growth, invasion and metastasis.

1.12 Phagocytosis by Epithelial Cells: Cell Sipping

This is a process in which cell membranes and/or fragments of cytoplasm can be exchanged between cell types—a non-mutational process with the potential to profoundly affect function, including neoplastic progression. It has been investigated in a number of cell lines, not so far those derived from HNSCC and could prove an important additional line of research (David et al. 2012). Transfer of mitochondrial function, and even whole mitochondria in this way, is recently recognised phenomenon (Berridge et al. 2016).

1.13 Microbial Interactions: Fungal, Bacterial and Viral

It is well known that certain fungal infections are associated with HNSCC. e.g. Oral leukoplakia with demonstrable Candidal hyphae embedded in the epithelium, have a higher risk of malignant transformation (Alnuaimi et al. 2015). This may be explained, in part, by the ability of candidal enzymes to produce potent carcinogenic nitrosamines and acetaldehyde, to provoke inflammation and divert the immune response (Krogh et al. 1987; Ramirez-Garcia et al. 2014).

A heavy bacterial load in the mouth, and by extension elsewhere in the upper aerodigestive tract, contributes to carcinogenesis, predominantly via generation of acetaldehyde (Moritani et al. 2015). There have been a number of recent studies reporting associations of periodontal disease with oral cancer (Yao et al. 2014; Javed and Warnakulasuriya 2016), a recent meta-analysis determined an odds ratio of 3.53 [95% CI (1.52–8.23); $P = 0.003$] for this association. There is new interest, with the advent of next generation sequencing methods, in describing the bacteriome (Wang and Ganly 2014) associate with progression of HNSCC. Sahingur and Yeudall (2015) hypothesise, in a recent review, that chemokines liberated by epithelium in the presence of a dysbiotic oral flora might trigger an oncogenic and periodontopathic cascade, including potentiation of bone resorption. This field of research has much further to go before it will reveal steps in molecular pathways amenable to intervention and inhibition of progression of the neoplasm.

A dysbiotic oral microflora triggers inflammatory processes in the oral epithelium. Release of chemokines, among other molecules, results in progression (or suppression, in some cases) of the inflammatory process and stimulation of both

innate and adaptive immune responses through recruitment of cellular mediators. Persistent inflammation extends deeper into the tissues, subsequently leading to osteoclast activation and subsequent destruction of alveolar bone. Multiple chemokines involved in the periodontal inflammatory process may stimulate their cognate receptors present on normal, dysplastic, or malignant epithelial cells, deregulating cellular growth, and promoting the motile phenotype. Pro-angiogenic chemokines, such as IL-8 and CXCL5, act upon endothelial cells to promote neovascularization of developing tumors.

Oncogenic viruses are discussed elsewhere.

1.14 Intra-epithelial Cell Populations: Langerhan's Cells, Melanocytes, Mast Cells, Merkel Cells and Their Role in the Neoplastic Process

1.14.1 Langerhans Cells (LCs)

These dendritic cells, located comparatively high above the basement membrane in all stratified squamous epithelia, are the main antigen processing cells for antigens which come into contact with such surfaces. They are thus fundamental to effective immune responses, and conversely to hypersensitivity reactions. As in much biology this is another ying/yang situation. LCs process polycyclic aromatic carbohydrates to a more potent carcinogenic form, which promotes HRAS mutations (Modi et al. 2012). The density of LCs is increased in oral leukoplakias with epithelial dysplasia, and again in oral SCC, indicative of enhanced immune surveillance, (Ohman et al. 2012) but this is not necessarily protective against neoplastic progression. Indeed other studies have shown the reverse: namely that LC density falls with increasing dysplasia and grade of (oral) cancer (Upadhyay et al. 2012). It is thus probable that the relevance of changes in LC density differ according to both the aetiology of a given patients carcinoma and of his/her immune *functionality* ab initio. A recent in vitro study has shown that an effect of the E6 oncogene of high risk HPVs in epithelial cell lines is to block the differentiation of monocytes into LCs (Lijima et al. 2013). LC vaccines are being explored for cancer therapy (Yanofsky et al. 2013). It is evident that case selection for this approach is critical.

1.14.2 Melanocytes

Malignant melanoma arising from the mucous membranes of the H&N is comparatively rare, but always carries a grave prognosis. The biology is distinctly different to HNSCC and is beyond the scope of this chapter.

1.14.3 Merkel Cells

These low level clear cells in skin and mucous membranes are associated with sensory nerve endings and respond to light touch. Merkel cell carcinoma (MCC) is a rare but dangerous skin cancer that arises most often in fair-skinned individuals over age 50. Most cases arise in sun-exposed skin of the head and neck and, more rarely, from the mucous membranes of the mouth or other upper aerodigestive tract sites (Lewis et al. 2010). Merkel cell carcinomas have a high propensity for regional and distant metastases, and recurrences are frequently seen. In all cases metastases to head and neck lymph nodes is an early and sinister sign, complicating staging and treatment (Pellitteri et al. 2012). In 2008 it was discovered that MCC is strongly associated with Merkel cell polyomavirus (MCPyV) (Feng et al. 2008), but this does not seem to be relevant to the more common forms of HNSCC.

1.14.4 Mast Cells

The role of mast cells in carcinogenesis and progression of neoplasms is double-edged. Mast cells today are regarded as important components of the immune system. They secrete a very wide array of cytokines and chemokines which promote both angiogenesis and inflammation. Clearly the former assists growth of the neoplasm. As already stated, inflammation can both promote and inhibit a neoplasm. Further work is needed to understand where, on the balance of such activities, a particular cancer in a particular patient is at any particular time. Effects are likely to vary in different parts of the primary neoplasm and in metastases, at different times. That said, mast cell infiltrates are not a conspicuous feature of most HNSCC and we are far from being able to manipulate mast cell behaviours to the advantage of the host. Current knowledge in this area is comprehensively reviewed by Khazaie et al. (2011).

1.15 Maintenance of Architecture of Stratified Squamous Epithelia, and Derangements in Cancer

The degree of success in maintaining architecture is seen by the degree of differentiation of each and every SCC. The generalisation that well-differentiated lesions are less aggressive than poorly differentiated or anaplastic neoplasms, going back to the classic work of Broders in the 1930s, (Broders 1941) holds true and has been developed by more sophisticated grading schemata (Lindenblatt et al. 2012) which take account not only of the epithelial component, but also of changes in stroma

(Johnson 1977) including the nature and intensity of the host immune/inflammatory response (discussed below). It is well recognised today that it is the character of the advancing front of the neoplasm which indicates the likely growth rate, invasiveness, and metastatic potential and which must be scored in written pathology reports: The various Colleges of Pathologists' pro-forma reporting aids are referred to below.

1.16 The Host Immune-Inflammatory Response to a Neoplasm

It was long thought that poor oral hygiene and chronic trauma, and the associated chronic inflammatory response, helped to promote oral/H&N cancer, the evidence coming from clinical and epidemiological studies (Zheng et al. 1990). Recent studies also confirm this (Bektas-Kayhan et al. 2014); These studies are reviewed by Gupta and Johnson (2014).

1.16.1 Good Inflammation Versus Bad Inflammation

On the other hand we have been conditioned to think of inflammation as a separate disease process from that of neoplasia. We have also been conditioned to think of inflammation, the fundamental defence reaction of vascular tissues to injury, as a good thing. Certainly if inflammation is intense, or prolonged, by-stander tissue injury is inevitable, but an inflammatory response to a developing or progressing carcinoma has been regarded as a beneficial host defence. This depended on the nature of the host immune/inflammatory response and, if this was an intense cell-mediated immune response, some early studies showed that histological evidence of this was a marker of relatively good prognosis (Johnson 1976): note that this feature is now a part of Pro-Forma Reporting in Cancer Diagnosis for H&N neoplasms (e.g. Royal College of Pathologists of Australasia 2014; Royal College of Pathologists 2014).

There is a rich cytokine milieu produced by the inflammatory infiltrate associated with most cancers, which interact to promote both proliferation and movement of malignant cells or, depending on concentration and time, may inhibit the neoplasm (see Fig. 1.13). Interestingly Woodford et al. (2014) have reported that whilst this is often prominent in premalignancy (now referred to as OPMD), it may subside when lesions progress to SCC (Figs. 1.14 and 1.15).

Although it is outside the scope of this chapter to review the literature comprehensively, there is growing evidence that non-steroidal anti-inflammatory drugs,

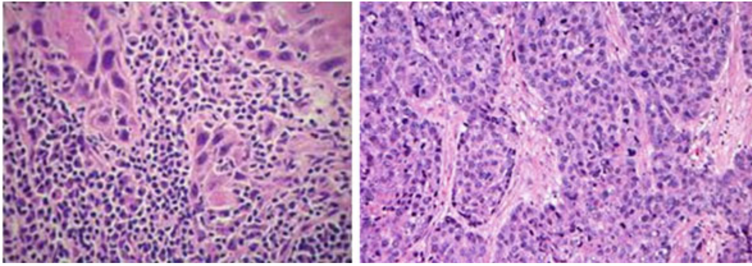

Fig. 1.13 Host inflammatory response to squamous cell carcinoma (courtesy N.W. Johnson)

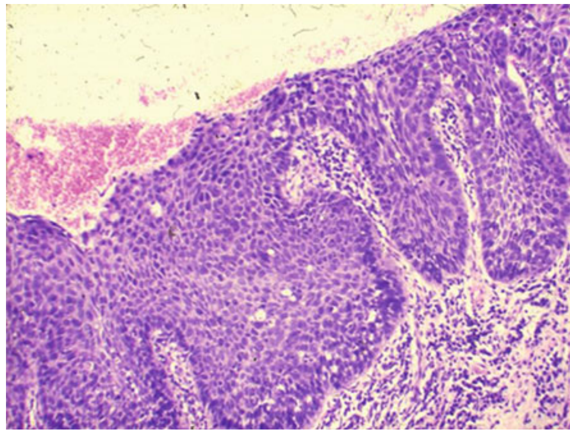

Fig. 1.14 Severely dysplastic oral mucous membrane which has elicited a strong lymphocytic response in the lamina propria (courtesy N.W. Johnson)

particularly Cox-2 inhibitors, including aspirin, can reduce the risk of malignant transformation in a range of dysplastic epithelial conditions, e.g. Barrett's oesophagus and in colon polyps, and reduce the incidence of skin, breast and colon cancer. This has to be balanced against increased risk of bleeding, including haemorrhagic stroke. Many clinical trials are underway, but the formal level of evidence is at present quite low. A collation and analysis of extant systematic reviews by an international consortium concludes that prophylactic aspirin use for a minimum of 5 years at doses between 75 and 325 mg/day appears to have a positive benefit-harm profile (Cuzick et al. 2015).

1.17 The Development of Tumour Stroma

Since the pioneering work of Sir Peter Medawar in the 1930s, for which he won a Nobel Prize, we have known of essential interactions between epithelial behaviour—including type and pattern of differentiation—and the connective tissues associated thereto. In the case of skin and mucous membranes, this relates to dermis and

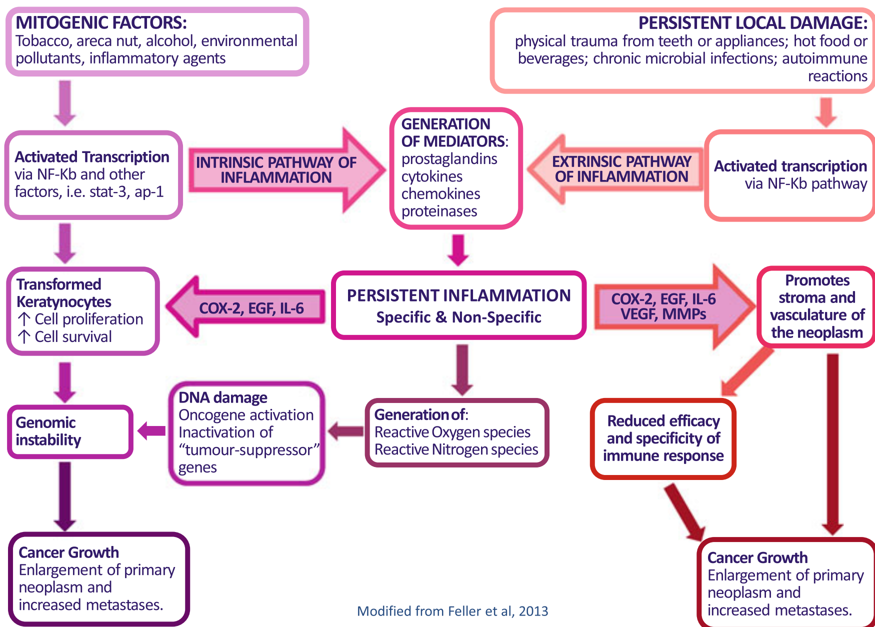

Fig. 1.15 Role of inflammation in cancer (modified from Feller et al. 2013). Figure re-drawn by Dr Luca Licheri

lamina propria respectively. During much of embryogenesis and in the maintenance of adult tissue, the nature of the connective tissue determines the type and behaviour of the epidermis/epithelial layer.

This is a major argument against the value of experiments with epithelial cell lines in cancer studies, only partly accounted for by use of co-culture with feeder fibroblasts and organotypic cultures!

The desmoplastic response seen in many solid epithelial neoplasms is but one sign. The developing stroma in most epithelial neoplasms has, naturally, a major component of fibroblasts and it is usual for these to develop markers of myofibroblasts, such as such as vimentin, α-smooth muscle actin, smooth muscle myosin, desmin, calponin, and α-integrin (Khazaie et al. 2004; Bhowmick and Moses 2005). Signalling by cytokines, other growth factors (GFs), matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs) takes place. The observation that silencing mutations on TP53 and PTEN, both of which are defined as tumour suppressor genes (TSGs), can be found in the fibroblast population but not in the epithelial cells in breast cancer, is solid evidence for drivers of the neoplasm coming from the stroma (Kurose et al. 2002) (Figs. 1.16 and 1.17).

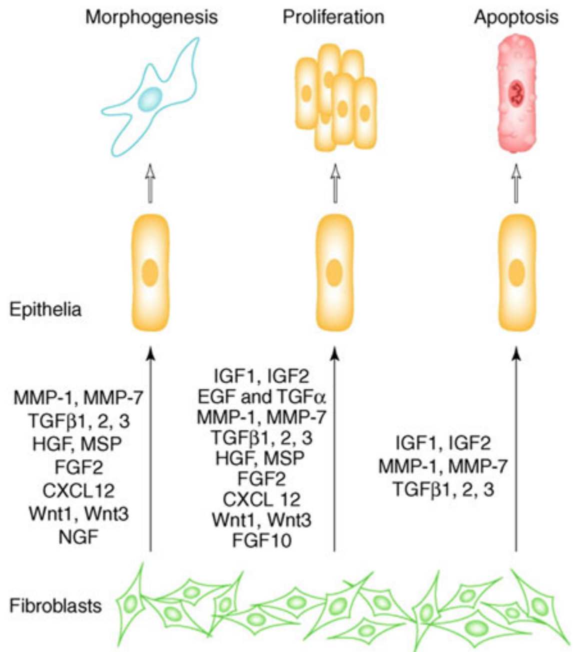

Fig. 1.16 The range of signals from stromal fibroblasts thought able to influence epithelial differentiation proliferation and death in epithelial neoplasms (reproduced from Bhowmick and Moses (2005) published in Curr Opin Genet Dev, NIH Public Access; Reproduced with permission)

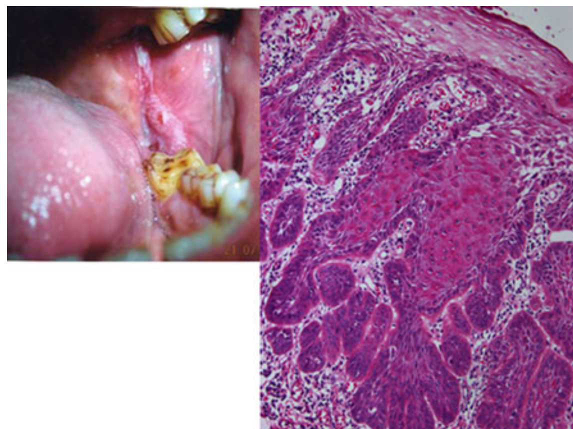

Fig. 1.17 Distinctive speckled leukoplakia in a 58 years old male executive with moderate alcohol and tobacco (Gutkha) chewing habits for 15 years. This early invasive SCC has evoked considerable angiogenesis and inflammatory infiltrate (courtesy of Dr. Vinay Hazarey, Nagpur, India)

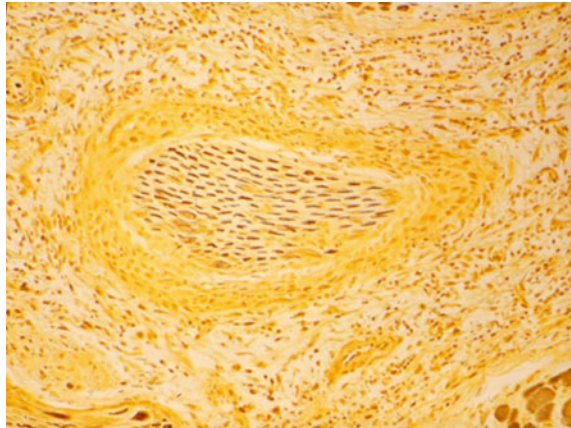

Fig. 1.18 Neural spread of a carcinoma: a ring of malignant keratinocytes encompasses this nerve bundle. Some muscle fibres appear bottom right (courtesy N.W. Johnson)

1.18 Metastasis: Local and Distant; Soil and Seed; Detection of Circulating Neoplastic Cells

1.18.1 Nerve Sheath Permeation

Amongst cancers of the head and neck, permeation along nerves is a particular property of adenoid cystic carcinomas, but can be seen with SCC (Fig. 1.18). It is a sign of poor prognosis and recurrence following surgery. There have been few investigations of molecular mechanisms and it is simplistically assumed that space between nerve bundles and nerve sheath connective tissues provide a line of least resistance.

1.18.2 Blood Borne Metastasis

Blood borne metastasis is dependent on neoplastic keratinocytes entering the blood stream, either directly within the infiltrating primary neoplasm, or via the thoracic duct after circulating in the lymphatic system, such cells presumptively coming from either the primary site, or a lymph node metastasis.

There is a body of work with H&N cancer patients in which peripheral blood has been screened for "circulating tumour cells" (CTCs), their presence inevitably being a marker of poor survival. Such studies require the collection of ~5–15 ml of peripheral venous blood and the selection of CTCs using presumed characteristic markers of such cells: nucleated cells containing cytokeratins, often vimentin, and either EGFR, CD44, or N-cadherin cell surface receptors: most of these are characteristic epithelial cell markers.

However, if EMT is fundamental to the process, such cells may express few epithelial markers, and it is logical to screen for nucleated (non-normal myeloid[1]) cells with markers more characteristic of EMT. This approach has been used by Balasubramanian et al. (2012) who show the presence of presumed CTCs in blood samples which are cytokeratin negative.

1.18.3 Mechanisms of Bone Invasion by HNSCC

Cancers which abut bone, most commonly the mandible from primary neoplasms of the floor of mouth, buccal sulcus or retromolar trigone, can invade by either an erosive process on a broad front, or by infiltrating through marrow spaces. The latter are obviously more dangerous and treatment requires wide excision of bone, making rehabilitation more challenging (Shaw et al. 2004). At a cellular and molecular level there is bi-directional signalling between cells of the cancer, the stroma and bone which enhance resorption but also provide pathways for intervention (Quan et al. 2012). We have shown that inhibition of MCP-1 (Monocyte chemotactic protein-1) may have such a role (Quan et al. 2014).

At (A) in Fig. 1.19, proteases, including matrix metalloproteinases (MMPs) and cathepsins help to degrade the matrix of bone which facilitates the entry of cancer cells into bone. Cytokines produced by the neoplasm stimulate production of RANKL (Receptor Activator of Nuclear factor kappa-B ligand, a TNF ligand superfamily member) by osteoblasts, which increases osteoclast function. At (B), cytokines/interleukins, chemokines and parathyroid-related proteins directly stimulate osteoclast function. At (C) growth factors are liberated by active osteoclasts which promote growth of malignant keratinocytes (Figure courtesy of Dr. J.J. Quan).

1.19 "Tumour" Immunity

The distinction between inflammation which inhibits, and that which promotes HNSCC, has been discussed above. The effect depends on the nature of the inflammatory process and on the stage in carcinogenesis, the degree of neoplastic progression, and the extent of metastasis.

Inflammation and immune response are a continuum. Acute inflammation, provoked by microbes or trauma, is a fundamental defence reaction and is characterised by vascular dilatation, increased capillary permeability and infiltration of granulocytes from capillary blood. Such a reaction, especially the cellular

[1]Note however the discussion in the section on EMT that myeloid markers may be a property of EMT and/or stem cells of an epithelial cancer.

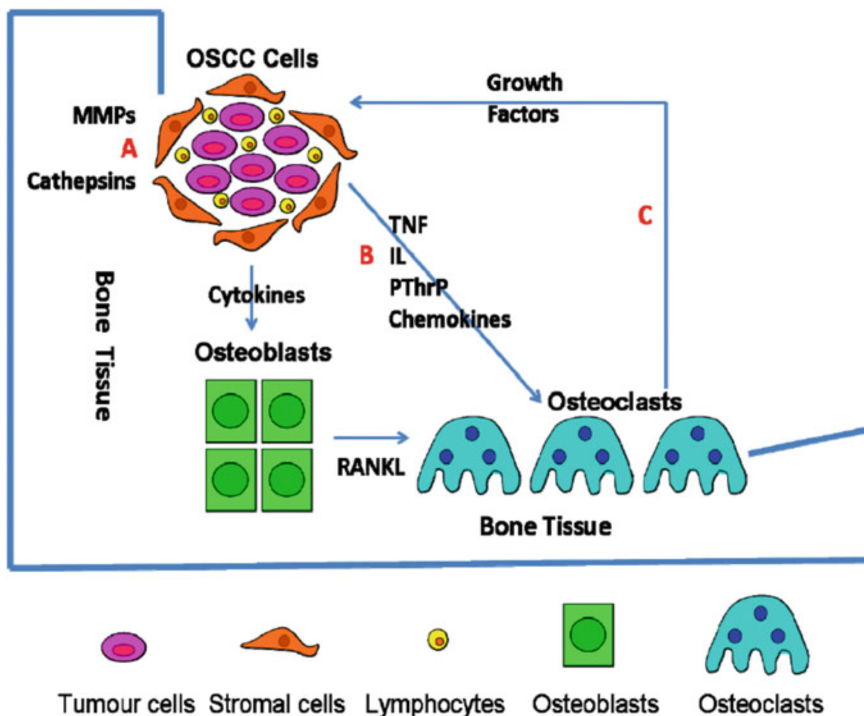

Fig. 1.19 Molecules released from malignant keratinocytes themselves promote bone invasion by stimulating osteoclastogenesis

component, is also the mechanism of innate immunity: a non-specific response to a foreign body, tissue, cell or molecule. This plays little role in HNSCC but an indication that the by-stander tissue injury aspect of acute inflammation can be effective is seen in the partial resolution of small melanomata on skin after injecting non-pathogenic bacteria or bacterial products, such as Corynebacteriae of BCG from Mycobacterium bovis, though much of the effect relies on development of later cell-mediated immunity. Such potentiation of B-cell and of T-cell immunity, with antineoplastic effects, was discussed at a Novartis Foundation Symposium in the 1970s but has received little attention since. This can be mediated by Toll-like receptors (TLR) (Rich et al. 2014). An increased expression of certain TLRs was found to help in the carcinogenic process through inducing inflammation (Kahn et al. 2016) (Fig. 1.20).

Neoplastic progression is accompanied by a progressively failing immune system, ultimately resulting in anergy.

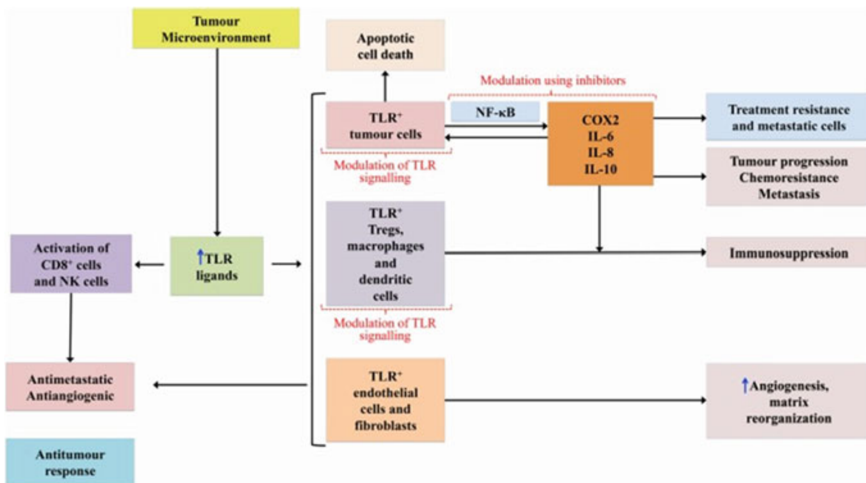

Fig. 1.20 The central, but bidirectional role, of TLRs in SCC ("Figure as originally published in Rich et al. (2014)". Reproduced with permission from Rich et al. (2014))

1.20 Nutrition and Cachexia

Cachexia (from the Greek κακός kakos "bad" and ἕξις hexis "condition") is loss of weight, muscle atrophy, fatigue, weakness, and significant loss of appetite. It is common in advanced cancer and contributes to poor immune response/anergy. Both preoperative and postoperative imaging to detect muscle loss has prognostic value in HNSCC. Recent evidence has improved our understanding of how this interacts with stress, and upregulating of the sympathetic nervous system which down regulates the immune response to the neoplasm (Repasky et al. 2015). It is not easily reversed, but expert management of the patient's nutrition, e.g. by maintaining high blood levels of amino acids is beneficial (Laviano et al. 2015).

1.21 Paraneoplastic Diseases: Local and Systemic

Paraneoplastic syndromes are most commonly associated with haematological and lymphoid neoplasms (notably Castleman's disease) and predominantly affect the central nervous system. Paraneoplastic pemphigus is of interest to head and neck oncologists because the lesions often present in the mouth or elsewhere in the upper aero-digestive tract (Yong and Tey 2013). Head and Neck malignancies may occasionally produce paraneoplastic diseases of a range of body systems (Chapireau et al. 2010; Toro et al. 2010).

1.22 Different Morphotypes of Squamous Cell Carcinoma: Structural and Behavioural Differences

Space does not permit a detailed account of the recognised subtypes of H&N SCC, which are well described in textbooks of H&N pathology. There is relatively little information as to whether or not there are significant differences in molecular pathways driving these different types of HNSCC and which thus might be differentially targeted in individualised patient therapy. Such thinking returns us to the fundamental need to identify key drivers in "*the unique biological event in the unique host*". The following types are recognised: Conventional; Verrucous carcinoma; Basaloid squamous cell carcinoma; Papillary squamous cell carcinoma; Spindle cell carcinoma; Acantholytic squamous cell carcinoma; Adenosquamous carcinoma; Carcinoma cuniculatum; Undifferentiated.

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Chapter 2
Cancer Stem Cells—Biopathology with Reference to Head and Neck Cancers

Miguel Ángel González Moles

Abstract Researchers have proposed that tumor development is based exclusively on the activity of cancer stem cells (CSCs), the so-called CSC hypothesis. This new model of carcinogenesis may offer insights into the high mortality of oral cancer, the poor response to treatment, and the elevated risk of multiple tumors in patients with oral squamous cell carcinoma (OSCC). Greater knowledge is needed of the molecular pathways that participate in maintaining the stem cell (SC) state in order to understand the mechanisms involved in tumor and metastasis development. This endeavor requires the development of specific markers to identify SCs and CSCs in tissues and to determine topographic relationships with their lineage. This chapter provides an update on the literature related to stem cells and oral cancer, centering on the CSC hypothesis in this context and on progress in the identification of oral stem cells.

2.1 Introduction

In the conventional clonal evolution model of carcinogenesis, an accumulation of genetic and epigenetic changes can have an impact on any oral epithelial cell, producing a clone with proliferative advantages that can develop invasiveness. According to the recently proposed cancer stem cell (CSC) hypothesis, only long-surviving cells, such as stem cells (SCs), can accumulate sufficient oncogenic alterations for carcinogenesis to be triggered. Hence, CSCs (transformed SCs) would be involved in the onset, progression, and spread of the tumor. If this is the case, the poor outcomes of oral cancer therapies may be attributable to their targeting of the whole tumor mass rather than specific cells responsible for its development and growth. On the other hand, CSCs have been attributed with molecular defense mechanisms and other features that may protect them against

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_2

conventional radio-chemotherapeutic treatment (Maitland and Collins 2005; Wicha et al. 2006). Targeted therapies that eliminate the CSC population offer the potential for cure. Given this promise, it is not surprising that the CSC hypothesis has attracted so much attention in recent years (Thomas et al. 2006).

There is therefore a need to develop novel therapeutic strategies for oral cancer that target CSCs. This task requires a greater understanding of the molecular pathways that maintain stemness. This chapter reviews current knowledge on SCs and CSCs in oral cancer, analyzing the protective anti-cancer mechanisms of normal stem cells and exploring techniques to distinguish normal from malignant SCs.

2.2 Stem Cells

SCs are characterized by their self-renewal capacity and their ability to differentiate into any type of mature cell (Reya et al. 2001; Shakib et al. 2011). Embryonic SCs can differentiate into cells of the three germinal lines to generate all types of tissue (Martin 1981; Evans and Kaufman 1981), while adult SCs are undifferentiated cells that only differentiate into the cell types present in the host tissue, which they can then regenerate (Zhang et al. 2012). The CSC is a recently proposed type of SC that differentiates in an aberrant manner and can produce tumor cell populations that are phenotypically different. According to the CSC hypothesis, CSCs drive tumorigenesis and tumor growth. However, knowledge of the proportions of CSCs and normal SCs in healthy or tumor tissue is hampered by the lack of specific markers of SCs and CSCs, especially in oral and head and neck squamous cell carcinomas (HNSCCs) (Boman and Wicha 2008). It was recently reported that an HNSCC contains less than one CSC for every 2500 cells (Ishizawa et al. 2010).

Their capacity for self-renewal allows SCs to persist throughout the life of the individual. They must be able to renew and maintain a balance between self-renewal and differentiation, preserving tissue homeostasis (Reya et al. 2001). If cancer is indeed a regulatory disorder of SC self-renewal, it is important to determine the molecular mechanisms that regulate normal SC self-renewal in order to understand the processes underlying tumor cell proliferation.

2.3 Spatial Ordering of Stem Cells and Progeny in Oral Epithelium in Relation to Antitumor Mechanisms

Normal SCs represent a small proportion of the cells in the oral epithelium, and their proliferation rate is low (Boman and Wicha 2008). In order to maintain the epithelial structure, there is consequently a need for a proliferative hierarchy with a certain spatial ordering. According to the hierarchical model of oral epithelium proliferation, in which the SCs divide asymmetrically, (Fig. 2.1), a normal SC

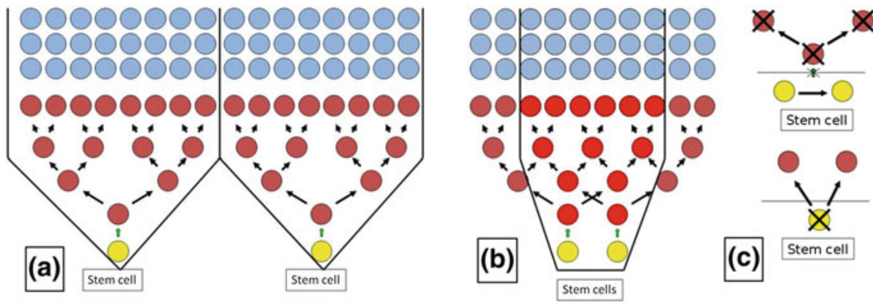

Fig. 2.1 **a** At each division, the normal SC (*yellow circles*) generates an identical SC and an ATC. The ATC (*brown circles*) divides 3–5 times until the terminal differentiation of all daughter cells. This asymmetric hierarchical proliferation predicts a theoretical spatial ordering of the progeny as inverted pyramidal structures (proliferative units—within *black lines*). **b** Areas of intersection (*red circles*) with neighboring proliferative units, in which the epithelial renewal would depend on the coordinated and alternating activity of different SCs. **c** above: symmetrical division of an SC giving rise to two SCs; below: Symmetrical division of an SC giving rise to two ATCs with loss of the SC.)

persists at each division and generates an amplifying transitory cell (ATC). The ATC can divide three to five times until the terminal differentiation of all daughter cells (Potten 1981; Ghazizadeh and Taichman 2001; Janes et al. 2002). The self-renewal capacity of ATCs is lower than that of SCs, and their principal role is to increase the number of differentiated cells resulting from the single division of a SC. Thus, if an ATC can divide three times before terminal differentiation, one SC would only have to divide once to generate one SC and eight ATCs for terminal differentiation. Accordingly, the oral epithelium contains three compartments: basal SC compartment; parabasal ATC compartment; and a more superficial epithelial compartment in which cells undergo terminal differentiation. This asymmetric division pattern may be an important antitumor protection mechanism, given that the ATC population is responsible for the greatest proliferation, carries the highest mutation risk, and finally develops terminal differentiation and desquamation (Janes et al. 2002) (see below).

Oral epithelial SCs can also divide symmetrically, generating two ATCs (losing the existing SC) or two cells with SC properties. This symmetrical division pattern may have evolved for the recovery of basal SC populations lost through traumatic or other lesions of the epithelium (Mackenzie 2006). The asymmetric hierarchical proliferation may predict a theoretical spatial ordering of the progeny of an oral SC as inverted pyramidal structures or “proliferative units” (Fig. 2.1). The vertex of the unit is one SC in the bottom stratum; the next stratum contains one ATC, followed by two, four, and eight ATCs in progressively higher strata, followed by a stratum of non-replicative cells undergoing differentiation. The areas between proliferative units would be pyramidal; in the usual ki-67-assessed epithelial proliferation pattern, however, parabasal proliferative areas are linear rather than pyramidal (Fig. 2.2). This may be attributable to the ability of new ATCs to expand laterally,

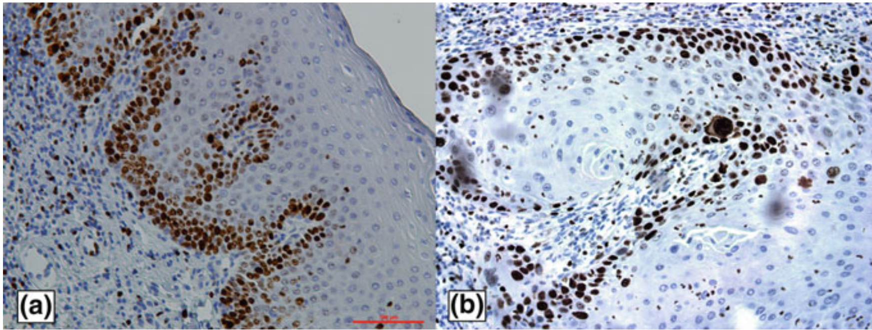

Fig. 2.2 **a** Although areas between proliferative units are predicted to be pyradimidal, the usual ki-67-assessed pattern of epithelial proliferative cells reveals linear rather than pyramidal parabasal proliferative areas (immunohistochemical technique, original magnification 20×). **b** Proliferative cells occupy basal and parabasal layers in premalignant and malignant epithelia (immunohistochemical technique, original magnification 20×)

occupying parabasal layers, meaning that the progeny of ATC division does not always pass to more superficial layers. This pattern would be more similar to the columnar organization observed on the dorsal skin of mice (Janes et al. 2002). In a human skin model developed by Janes et al. (2002), ATCs form a network around clusters of SCs containing around 40 cells. In common with our model, the assumption was made that each proliferative unit is maintained by more than one SC and that ATCs migrate laterally in parabasal layers. The linear rather than pyramidal proliferative pattern may also indicate areas of intersection between proliferative units and neighboring units, where epithelial renewal would be governed by the coordinated and alternating activity of different SCs, with ATCs proliferating in the parabasal layer responsible for epithelium renewal. In fact, epithelial renewal areas influenced by more than one SC may represent a further antitumor mechanism, allowing a reduction in the proliferation of each SC while maintaining epithelial homeostasis. It is not easy to determine the number of SCs, but all basal cells are very unlikely to be SCs. The nature of non-SC basal cells and their function in maintaining epithelial homeostasis has not yet been elucidated; however, they may be intermediate cells between SCs and ATCs with a low proliferation rate, possibly playing a role in the structural maintenance of SCs in the basal epithelial layer, as proposed in the human epidermis (Jensen et al. 1999).

2.4 Cancer Stem Cells

It is currently thought that a small group of tumor cells, known as CSCs, have the ability to self-renew and generate a phenocopy of the original tumor (Clarke et al. 2006; Sampieri and Fodde 2012), being progenitors of tumor bulk cells and driving tumor growth.

The idea that CSCs are responsible for tumorigenesis derives from the heterogenous composition of tumors, including OSCCs. Histologic studies reveal distinct areas of tumors with different degrees of differentiation; while genetic studies show a similar heterogeneity, with areas that differ in gene expression and therefore in protein expression; the cells in a tumor differ in proliferation rate and capacity to generate new tumors. As already noted, it is proposed that a small cell population of CSCs with a distinctive phenotype is responsible for the growth of a new tumor (Reya et al. 2001; Bánkfalvi et al. 2002; Tremmel et al. 2003; Pardal et al. 2003; Costea et al. 2006; Zhou and Jiang 2008; Visvader and Lindeman 2008; Märgäritescu et al. 2011). Their existence was also brought to mind by the similarity in histologic profile and proliferation pattern between well-differentiated tumors and their epithelium of origin. The former are commonly organized in three compartments, as in normal epithelium (basal CSC, ATC, and innermost differentiated cell compartment). This similarity in proliferative hierarchy with non-tumor oral epithelia suggests that tumor growth is maintained by a single type of tumor cell, the CSC.

Bonnet and Dick (1997) found that a small group of tumor cells with CD34+/CD38 phenotype in human acute myeloid leukemia (AML) was able to generate AML when transplanted to NOD/SCID mice, but the other tumor cells were not. They proposed that the human AML is organized as a hierarchy of cells derived from primitive hematopoietic cells. Likewise, breast cancer tumors were generated in NOD/SCID mice by a small group of CD44+/CD24− cells but not by the 100-fold more numerous CD44− cells (Al-Hajj et al. 2003). CSCs have subsequently been evidenced in other solid tumors (Singh et al. 2003; Fang et al. 2005; O'Brien et al. 2006; Ho et al. 2007; Hermann et al. 2007; Yang et al. 2008) including HNSCC and OSCC, in which only a small subset of CD44+ cells was able to generate a tumor (Zhang et al. 2012).

2.5 Stochastic Model of Tumor Origin and the CSC Hypothesis

According to the stochastic model, malignant transformation is due to the random mutation of any cell, generating a tumor through the clonal progression of mutant cell progeny that possess a proliferative advantage and produce a genomic instability that leads to the accumulation of epigenetic/genetic events and the selection of progressively more aggressive subclones (Nowell 1976; Wicha et al. 2006; Campbell and Polyak 2007). Development by these subclones of different phenotypes and proliferative capacities would be responsible for tumor heterogeneity. In this model, cancer is considered a proliferative disease (Shakib et al. 2011).

The CSC hypothesis, supported by the aforementioned experimental evidence (Nowell 1976; Lapidot et al. 1994; Bonnet and Dick 1997; Al-Hajj et al. 2003; Singh

et al. 2003; Singh et al. 2004; Kim et al. 2005; Fang et al. 2005; Collins et al. 2005; O'Brien et al. 2006; Campbell and Polyak 2007; Prince et al. 2007; Dalerba et al. 2007; Hermann et al. 2007; Yang et al. 2008) has two components. The first component is related to the cell origin of tumors and the increased risk of accumulating multiple mutations during the long life of SCs. The malignant transformation of a normal cell is estimated to require 3–6 oncogenic events (Kinzler and Vogelstein 1996; Hahn and Weinberg 2002). A genetic model of progression was proposed in OSCC, correlating summative genetic alterations with phenotypic progression to malignancy (Califano et al. 1996; Califano et al. 2000; Gollin 2001) and indicating that the accumulation of sufficient mutations to develop OSCC would only be possible in cells with a long life. Furthermore, SCs and CSCs both possess self-renewal capacity, and self-renewal dysregulation is an early and key event in carcinogenesis. The self-renewal machinery is activated in normal SCs, and maintenance of this activation would appear more likely in comparison to a de novo activation in a more differentiated cell (Sawyers et al. 1991; Sell and Pierce 1994; Reya et al. 2001). The suggestion is that CSCs appropriate the self-renewal machinery in normal SCs. Animal studies have implicated dysregulation of the molecular pathways involved in self-renewal (Wnt, Notch and Hedgegog) in tumorigenesis (Wicha et al. 2006). Notch and Hedgehog activation enhances self-renewal in hemopoietic stem cells (HSCs) (Varnum-Finney et al. 2000; Karanu et al. 2000; Bhardwaj et al. 2001) and is observed in leukemias and other cancers (Unden et al. 1996; Diévart et al. 1999; Nam et al. 2002; Nickoloff et al. 2003; Benson et al. 2004; Olsen et al. 2004; Karhadkar et al. 2004). The pool of HSCs and progenitor hematopoietic cells is augmented by Wnt, with its downstream activator ß-catenin, increasing the cell proliferation rate in human bone marrow (Austin et al. 1997; Van Den Berg et al. 1998), while keratinocyte proliferation was stimulated by increased ß-catenin and Wnt protein levels in culture (Reya et al. 2001). Activation of the Wnt pathway has been observed in colon, skin, and oral cancer (Gat et al. 1998; Liu et al. 2010; Iwai et al. 2010; Fujii et al. 2011; Ravindran and Devaraj 2012).

The second component of the CSC hypothesis is that CSCs are responsible for maintaining tumor growth, supported by findings that SC transplantation into NOD/SCID mice not only enhanced cell self-renewal but also promoted tumor growth through their aberrant differentiation capacity, reproducing the heterogeneity of the original tumor (Wang and Dick 2005).

If the CSC hypothesis is correct, normal SCs must possess powerful anticancer mechanisms to reduce the risk of malignant transformation. One mechanism may be their asymmetric division pattern, giving rise to ATCs for proliferation, differentiation, and desquamation and preventing the accumulation of mutations in ATCs that suffer initial oncogenic events. The rate of SC division is also low in asymmetric division, reducing the risk of mutation at each mitosis, and can be even lower if renewal areas are influenced by more than one SC. A further anti-cancer mechanism may involve the selective segregation of DNA in the asymmetric division of the SC, as observed in bowel and breast SCs, with secretion of the new synthesized DNA strand from the SC compartment into ATCs (Potten et al. 2002; Dontu and Wicha 2005; Mackenzie 2006). In fact, the genome of adult SCs must be

highly stable, because they must be maintained and preserved by DNA repair mechanisms over an individual's lifetime. Thus, a high incidence of cancer is observed in syndromes with DNA repair defects (e.g., ataxia telangiectasia and xeroderma pigmentosum) (Mackenzie 2006). Some results suggest that CSCs may undergo altered behavior, including therapeutic resistance as a result of chromosomal instability due to chromosome segregation defects (Kaseb et al. 2016).

It is also possible, although less likely, that CSC may originate from the fusion of an HSC with a differentiated epithelial cell, acquiring the self-renewal capacity of the HSC (Bjerkvig et al. 2005), producing genomic instability, and promoting the accumulation of oncogenic events. A further possibility is the fusion of an HSC with a mutated epithelial somatic cell, giving rise to a mutant cell with SC features that can accumulate further oncogenic events. The fusion of HSCs with epithelial cells has been demonstrated *in vitro* (Wagers and Weissman 2004) and in animal models of stomach cancer (Houghton et al. 2004) but not in OSCC. The dedifferentiation of a mature cell might also result in a CSC, with oncogenic events leading mature epithelial cells to retrieve their self-renewal capacity and lose their terminal differentiation capacity (Gat et al. 1998; Zhu and Watt 1999). These cells may acquire additional mutations that produce their transformation (Perez-Losada and Balmain 2003). However, the acquisition of SCs requires the reprogramming of differentiated cells, for which identity maintenance is essential; therefore, this reprogramming would require the involvement of powerful regulators of the transcriptional and/or epigenetic machinery (Abollo-Jiménez et al. 2010). Four transcription factors participate in reprogramming in oncogenesis, and the early inhibition of somatic genes involved in differentiation by c-Myc appears to be crucial. Histone deacetylase inhibitors can replace c-Myc in reprogramming (Huangfu et al. 2008; Iglesias-Linares et al. 2010), participating in suppression of the differentiated cell's gene expression program. The transcription factors OCT-4, Sox-2, Klf-4, and 4YTF become involved in reprogramming at a later stage (Abollo-Jiménez et al. 2010).

It was recently reported that differentiated cancer cells can become CSC-like via epithelial mesenchymal transition (EMT) , in which epithelial cells acquire mesenchymal characteristics under the influence of specific environmental stimuli and can invade surrounding tissues and spread to distant organs (see Chap. 1). EMT is promoted by the activity of transcription factors such as Snail, Twist 1, and ZEB 1 (Batlle et al. 2000; Yang et al. 2004; Mani et al. 2008; Sánchez-Tilló et al. 2010), which change epithelial cell polarity and repress E-cadherin expression, among other effects (Cano et al. 2000). EMT was found to be involved in the acquisition by differentiated cells of SC properties in breast (Morel et al. 2008) and nasopharyngeal (Xia et al. 2010) cancer and HNSCC, in which Bmi-1, another transcription factor involved in SC self-renewal, was induced by Twist 1 and E-cadherin expression was repressed (Park et al. 2004; Valk-Lingbeek et al. 2004; Widschwendter et al. 2007; Spivakov and Fisher 2007; Zhang et al. 2012). E-cadherin downregulation in suprabasal oral premalignant epithelia has been implicated in multiple tumor development, which may indicate a reprogramming process related to molecular mechanisms involved in self-renewal and the acquisition of CSC-like characteristics

by partially-differentiated oral epithelial cells (ATCs) (González-Moles et al. 2012a, b).

Finally, CSCs can be produced by neosis, a new type of cell division seen in cancers, in which DNA damage can generate senescent multinucleated cells that evade apoptosis, divide, and give rise to SC-like cells (Sundaram et al. 2004); their division produces mononuclear "Raju" cells with extended mitotic life and the capacity to generate transformed cell lines (Sundaram et al. 2004; Rajaraman et al. 2005), similar to observations in senescent oral keratinocytes in culture (Kang et al. 2000). Nevertheless, although multinucleate cells are common in OSCCs and premalignant epithelia, it is not known whether neosis can give rise to CSCs in OSCC (Costea et al. 2006).

Proliferative patterns observed by our group (González-Moles et al. 2010) in OSCC and non-tumor epithelia adjacent to invasive carcinomas (González-Moles et al. 2000; González-Moles et al. 2010) suggested different origins for CSCs and precancerous SCs. Basal proliferation can sometimes be observed in premalignant epithelia and tumor nests neighboring non-neoplastic epithelia. Given the basal localization of SCs, this may indicate that the usual asymmetrical division of normal SCs is replaced with a symmetric division, in which a premalignant SC or CSC generates two premalignant SCs or two CSCs that stay in the basal layer of premalignant epithelia or at the periphery of well-differentiated tumor nests, respectively. The over-accumulation of premalignant SCs would exceed the capacity of the basal layer, gradually leading to their occupation of parabasal layers. Likewise, the basal and suprabasal proliferation of CSCs can also produce an expansive growth in well-differentiated OSCC nests (Fig. 2.2). On the other hand, elevated basal and suprabasal proliferation rates in some epithelia might also be attributed to premalignant or malignant SCs that still proliferate asymmetrically but have suffered mutational events that enhance their proliferation rate. Asymmetric division of these CSCs would lead to a malignant transitory amplifying compartment that is able to differentiate, accounting for the maintenance of tumor nests with a similar appearance to that of normal or premalignant epithelia. Another frequent observation in premalignant epithelia is a scantly proliferative basal layer with highly proliferative parabasal cell layers (2–3 layers) (Fig. 2.2), which was associated by our group with multiple tumor development (González-Moles et al. 2010). This observation may indicate that the tumor does not only originate in normal basal SCs but can sometimes originate in ATCs, whose high proliferation rate may increase the mutation risk, altering the reprogramming process; this would allow ATCs to gain greater self-renewal capacity and maintain an elevated proliferation rate without completely losing their differentiation capacity, i.e., CSC-like characteristics. In the presence of genomic instability, this would increase the risk of new oncogenic events.

Proliferative cells are very occasionally observed in epithelium above the transient amplifying compartment, which may possibly correspond to the reprogramming of differentiated cells. The virtual absence of proliferative cells in superficial layers of normal epithelium corroborates the difficulty of reprogramming differentiated cells, which requires major molecular changes.

2.6 Identification of Stem Cells and CSCs

There is a lack of SC markers, limiting investigation of the participation of SCs in carcinogenesis. They can be only identified by analyzing their replicative behavior *in vitro* and detecting long-lived cells in tissues (Janes et al. 2002).

In *in vitro* clonal trials, keratinocytes in low-density cultures produce different types of colonies depending on their precursors. Large, compact, actively growing colonies are sometimes observed, known as holoclones (Barrandon and Green 1987), comprising small cells from individual founding cells that can be repeatedly passaged. Other keratinocytes divide a few times, lose their proliferation capacity, and develop terminal differentiation, producing abortive colonies (paraclones) of large flattened cells. Finally, keratinocytes can generate colonies (meroclones) of cells in a state of transition between holoclones and paraclones. Cells forming holoclones tend to be classified as SCs, while cells forming paraclones are considered ATCs. As in the normal epithelium, malignant keratinocytes can give rise to malignant holoclones that differ from malignant paraclones, comprising smaller, more adhesive, and more rapidly clonogenic cells. The behavior and expression patterns of markers of cells that make up malignant holoclones are similar to those of normal epithelial SCs and possess the essential characteristics of a CSC (Locke et al. 2005). The *in vitro* clonal assay is a reproducible method for identifying and isolating SCs and CSCs and evaluating their response to therapeutic agents (Janes et al. 2002).

Long-surviving cells in the oral mucosa can be identified by labeling and flow cytometry using β-1 integrin, α-6 integrin, CD71, E-cadherin, β-catenin, epithelial-specific antibody, and CD44 as surface makers, most of which show elevated expression in holoclones from OSCC cell lines. However, there are few reliable markers of oral SCs (Tudor et al. 2004). Many are also expressed by non-SCs, e.g., β-1 integrin (Jones et al. 1995) and CD44 (González-Moles et al. 2004), while some are intracellular and undetectable by flow cytometry (Kaur et al. 2004).

The most frequently applied method for identifying CSCs in culture is to use flow cytometry to detect cells able to excrete the vital DNA dye Hoechts 33342 (Goodell et al. 1996; Wang et al. 2007; Loebinger et al. 2008; Zhou et al. 2008; Sung et al. 2008; Zhang et al. 2009; Chen et al. 2009; Yajima et al. 2009; Song et al. 2010; Yanamoto et al. 2011), recording dye-retaining cells and a distinctive small non-dyed population of cells (side population [SP]) expressing SC markers. The capacity of SPs to excrete Hoechts 33342 dye depends on the cell membranous activity of the superfamily of ABC transporter pumps. The human ABCG2 member is considered a CSC marker (Seigel et al. 2005; Ho et al. 2007; Shi et al. 2008; Wang et al. 2009). SP cells have been identified in various tumors (Kondo et al. 2004; Hirschmann-Jax et al. 2004; Haraguchi et al. 2006; Chiba et al. 2006; Szotek et al. 2006) and in normal tissue (Shimano et al. 2003; Kim and Morshead 2003; Majka et al. 2005; Larderet et al. 2006). In HNSCC, SP cells are highly tumorigenic (Wang et al. 2007; Zhang et al. 2009; Song et al. 2010; Wan et al. 2010; Tabor et al. 2011), expressing SC markers (e.g., ABCG2, (Mack and Gires 2008; Song et al. 2010; Tabor et al. 2011), Bmi-1(Wan et al. 2010; Tabor et al. 2011), CD44, and

Oct4 (Zhang et al. 2009), and showing an abnormal Wnt signaling pathway(Song et al. 2010; Tabor et al. 2011). The SP population is highly variable in OSCC cell lines, ranging from 0.2 to 10% of the cancer cell population (Zhang et al. 2009).

However, these procedures are not suitable for routine use and do not enable the topographic localization of SCs in healthy or tumor tissue for evaluating their proliferative activity or spatial relationships with their progeny. Some promise has been shown by the following markers:

2.6.1 β-1 Integrin

β-1 Integrin is a potential oral SC marker, is expressed in basal keratinocytes and downregulated when cells leave the basal layer (Cotsarelis et al. 1999; Janes et al. 2002). Human keratinocytes can be classified by the rate of their binding with type IV collagen, the natural ligand of β-1 integrin, into two types. Fast-adhering keratinocytes resemble SCs (Jones et al. 1993; Jones 1996), whereas slow-adhering keratinocytes, with low levels of integrin β-1, behave as late ATCs (Jones et al. 1993; Jones 1996), suggesting that integrin β-1 is required to maintain the keratinocytes in an undifferentiated state (Adams and Watt 1989; Levy et al. 2000; Hombach-Klonisch et al. 2008). The main shortcoming of integrin β-1 as a SC marker is its lack of specificity, given that around 20–45% of basal keratinocytes show high integrin β-1 expression.

2.6.2 Transcription Factors Oct3/4, Sox and Nanog

Oct3/4 (Nichols et al. 1998; Niwa et al. 2000), Sox (Avilion et al. 2003) and Nanog (Chambers et al. 2003; Mitsui et al. 2003), play a fundamental role in maintaining the pluripotency and self-renewal of embryonic and adult SCs (Boyer et al. 2005; Loh et al. 2006; Campbell et al. 2007), promoting self-renewal through interaction with Stat-3, Hesx-1, and Zic-3, and signaling molecules TCF-3, FGF-2, and LEFTV2 (Boyer et al. 2005; Loh et al. 2006). Oct3/4 is regarded as one of the best indicators of stemness (de Jong and Looijenga 2006; Marynka-Kalmani et al. 2010). HNSCC cells forming holoclones express high levels of these factors (Lim et al. 2011), suggesting that cancerous cells expressing these factors have SC-like behavior (Zhang et al. 2012).

2.6.3 CD 133

The protein CD 133 (Miraglia et al. 1997; Yin et al. 1997) was used as a marker of HSCs (Yin et al. 1997; Chiou et al. 2008) and subsequently as a marker of epithelial

(Weigmann et al. 1997; Corbeil et al. 2000), neural (Uchida et al. 2000; Lee et al. 2005), prostate (Shepherd et al. 2008), and kidney (Bussolati et al. 2005) SCs. Studies in OSCC (Zhang et al. 2010) have demonstrated that a small proportion of tumor cells (1–3%) are CD133+ and are highly clonogenic and tumorigenic, showing increased resistance to chemotherapy (Zhang et al. 2010). CD133+ cells from OSCC form holoclones and possess self-renewal capacity (Chiou et al. 2008), while CD133 is expressed by CSCs in the Hep-2 laryngeal cancer line (Zhou et al. 2007; Wei et al. 2009). Therefore, CD133 may be a useful CSC marker in HNSCC and OSCC (Zhang et al. 2012), although some authors did not detect CD133+ cells in oral cancer (Märgäritescu et al. 2011).

2.6.4 CD44

CD44 (Aruffo et al. 1990; Screaton et al. 1992) provides anchorage for MMP-9, which is essential for metalloproteinase activity and may favor tumor invasiveness. CD44 was the first CSC marker to be used in breast cancer, and Prince et al. (2007) reported a group of CSC-enriched CD44+ cells in HNSCC that could be serially passaged in vivo, reproducing the original tumor. These CD44+ cells expressed high Bmi-1 levels and displayed self-renewal and differentiation capacities. However, questions have been raised about the value of CD44 as a CSC marker in OSCC because of its expression by a much larger number of tumor and normal oral epithelial cells (González-Moles et al. 2004; Mack and Gires 2008; Clay et al. 2010; Oliveira et al. 2011) than the small number of CSCs considered to be present in oral tissues. There is also debate about the usefulness of CD44 as a marker of OSCC progression and prognosis. Thus, some researchers associated higher CD44 expression with greater tumor aggressiveness (Okamoto et al. 2002; Chen et al. 2010; de Jong et al. 2010; Joshua et al. 2012), while others associated reduced or loss of CD44 expression with a negative prognosis (Sato et al. 2000; Carinci et al. 2002; González-Moles et al. 2002, 2004; Kosunen et al. 2007; Clay et al. 2010). The loss of CD44 expression was found by our group to be an early event in oral carcinogenesis, favoring the acquisition of invasive capacity by premalignant epithelial clones and associated with a greater thickness of tumor invasion, behaving as an independent predictor of poor survival. Association of the loss of a stem cell marker with a worse prognosis appears inconsistent with the notion that tumor growth depends on CSCs, although this may be explained by an ability of some CSC properties (e.g., expression of some adhesion molecules) to be lost and re-expressed, favoring invasion and the reestablishment of stable, cohesive metastatic colonies. This has been reported for the expression of E-cadherin adhesion molecule in EMT and in the mesenchymal-to-epithelial transitional stage (MET) (Kudo et al. 2004; Zhang et al. 2012). It may be possible to improve the value of CD44 as a SC marker by combining it with DHAL activity markers. DHAL, a family of intracellular enzymes, participates in cell differentiation, detoxification, and drug resistance through the oxidation of intracellular aldehydes

(Moreb et al. 1996; Magni et al. 1996; Sophos and Vasiliou 2003; Chute et al. 2006). DHAL1, the prototype of the family, is expressed in HSCs and hematopoietic progenitor cells (Magni et al. 1996; Chute et al. 2006), and in normal and malignant breast and lung SCs (Ginestier et al. 2007; Sullivan et al. 2010). Some studies in HNSCC have described ALDH-positive cells with CSC-like behavior and increased tumorigenic capacity (Chen et al. 2009; Clay et al. 2010; Krishnamurthy et al. 2010; Chen et al. 2011). It was reported that the specificity of CD44 as a CSC marker in HNSCC is increased by combining it with ALDH (Chen et al. 2009; Clay et al. 2010), and the production of a tumor in NOD/SCID mice was found to require the transplantation of tenfold fewer ALDH+/CD44+ cells in comparison to cells positive for CD44 alone (Prince et al. 2007). According to these findings, ALDH expression may isolate a subset of CSC-enriched CD44+ cells, although both markers are not always expressed in CSCs, and there is a small proportion of CSCs that are positive for ALDH and negative for CD44 (Clay et al. 2010).

2.6.5 E-Cadherin

E-cadherin binds to actin in the cytoskeleton through interaction with catenins (Hajra and Fearon 2002). The downregulation of E-cadherin, an invasion suppressor molecule (Vleminckx et al. 1991), predicts a worse prognosis (Hoteiya et al. 1999). E-cadherin underexpression was observed by our group in precancerous fields associated with multiple tumor development (González-Moles et al. 2012a, b). Loss of E-cadherin expression allows the release of ß-catenin, which acts as a transcription factor, activating the Wnt pathway (Takes et al. 2001). E-cadherin can be downregulated by hypermethylation of CDH1 promoter, enhancing the metastatic potential of hypermethylated clones. E-cadherin-methylated tumor cells may be focally dissociated at invasion fronts, spread to lymph nodes and then become demethylated, reacquiring E-cadherin expression (Kudo et al. 2004). Hence, methylation is reversible in OSCC progression, an important issue because E-cadherin may favor the growth of metastatic tumor cells nests by compacting cellular aggregates and activating apoptosis evasion pathways (Kantak and Kramer 1998). As noted above, E-cadherin downregulation is essential in EMT, promoting invasiveness and conferring tumor cells with SC properties (Yu et al. 2012), while E-cadherin recovery is important for MET. Hence, the state of a cell and its SC characteristics can be modified to gain an invasive or metastatic advantage. However, despite its role in the physiology and biopathology of SCs, doubts remain about the value of E-cadherin as a CSC marker in OSCC (Zhou and Jiang 2008), mainly because of its low specificity (González-Moles et al. 2012a, b).

A range of other molecules have been tested for CSC markers. These include CD147, CD97, CD117, Ep CAM, CK19 and ESA but have shown poor specificity for the identification of oral SCs (Hombach-Klonisch et al. 2008; Zhou and Jiang 2008; Richard and Pillai 2010).

2.7 Conclusions

The CSC hypothesis opens up a wide field for future research, and there is an urgent need to identify specific markers for the detection of cancer stem cells in routine laboratory tests. The availability of reliable markers would improve knowledge of the cell types that generate a tumor, their distribution in tissues, and their relationships with their progeny, allowing exploration of the prognostic relevance of their proliferative activity and invasive capacity. This type of research has potentially important therapeutic implications, given the possibility of designing effective therapies that specifically target CSCs.

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Chapter 3
Hypoxia in Head and Neck Cancer

Mahvash Tavassoli and Yae-eun Suh

Abstract Head and neck cancer is the sixth most common malignancy worldwide and a major cause of death from cancer, with a median 5-year survival of around 50% (Suh et al. 2014). Over 90% are squamous cell carcinomas arising from the epithelial cells that line the mucosal surfaces of the head and neck. Tumour hypoxia is an important negative prognostic factor for head and neck squamous cell carcinomas (HNSCC), associated with resistance to radiotherapy and decreased overall survival (Bittner and Grosu 2013). Therefore, tumour hypoxia is an important phenomenon in the management of HNSCC and has been the focus of studies to improve treatment response for many years. This chapter aims to discuss some of the key mechanisms affected by tumour hypoxia and review strategies under investigation towards better detection and modulation of tumour hypoxia in order to improve treatment response and survival of head and neck cancer patients.

3.1 Background

Tumour heterogeneity due to genetic and epigenetic variations within the tumours is an important contributor to phenotypic diversity of tumours and resistance to therapy (Catenacci 2014). In addition, tumour microenvironment influences phenotypic diversity or plasticity within the tumour (Fukumura and Jain 2007). Hypoxia is a fundamental biological phenomenon of solid tumours, which plays an important role in changing tumour microenvironment (Wouters et al. 2002). Hypoxia leads to cellular individuality and is associated with resistance to curative therapies and reduced survival in a number of tumour types including head and neck, pancreatic, cervical and prostate cancers (Buffa et al. 2010; Eustace et al. 2013). Tissue hypoxia occurs when the oxygen level in tissues falls below

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_3

physiological levels resulting from the inadequate supply of oxygen to meet demand (Hockel and Vaupel 2001). It arises in tumours due to the high metabolic demand of rapidly proliferating cancer cells, and due to disruption in the blood supply. Tumours develop aberrant and chaotic microvasculature, which are structurally immature and leaky, resulting in ineffective oxygen delivery (Vaupel 2004). Fluid accumulation and increased tumour interstitial pressure restricts and compresses intratumoural blood vessels, further reducing the blood supply. Hypoxia leads to increased risk of tumour progression, metastases, resistance to anti-cancer therapies and recurrence due to a wide range of hypoxia-related consequences, resulting in metabolic and genetic changes that promote an aggressive tumour phenotype (Vaupel and Mayer 2007). Hypoxia drives tumour progression by selecting clones that can best adapt to the stress of inadequate perfusion and nutrient deprivation, which then expand and develop into a more malignant phenotype.

Hypoxia has been extensively investigated in HNSCC and its association with poor outcome is well known. However, it is difficult to assess, as intratumoural hypoxia is heterogeneous and dynamic, due to continuing tumour and vessel growth, and constant fluctuations in blood flow. Different biological effects are triggered at different levels of oxygen partial pressure (pO_2) in tissues. There is no consensus for hypoxic thresholds in tumours but at $pO_2 < 25–30$ mmHg radiosensitivity progressively decreases (Hockel and Vaupel 2001; Rademakers et al. 2008), and at $pO_2 < 10–15$ mmHg changes in gene expression under the control of the hypoxia regulated transcription factor HIF-1 are demonstrated. At $pO_2 < 10$ mmHg Vaupel et al. (1994) demonstrated intracellular acidosis and adenosine triphosphate (ATP) depletion in murine fibroblasts and this level represents a critical threshold for energy metabolism. There is also decreased protein synthesis and oxygen consumption to increase tolerance to hypoxia. At $pO_2 < 1$ mmHg there is increased apoptosis, reduced oxidative phosphorylation and cells switch to glycolysis (also known as Warburg effect) to maintain adequate energy levels. Lower levels of pO_2 of 0.2–1 mmHg lengthen G_1 phase of the cell cycle or arrest cells in G_1 and anoxia causes immediate cell cycle arrest (Hockel and Vaupel 2001).

Importantly, hypoxia is a transportable property. Studies performed using patient derived xenografts with differing degrees of hypoxia demonstrated that the amount of hypoxia in the xenograft derived from hypoxic tumours had similar oxygen distribution to the patient's tumour. The hypoxic tumours grew more rapidly and when these human derived xenografts were serially propagated, they maintained the hypoxic feature (Chang et al. 2011). These findings suggest that the genetics of cancer influences the microenvironment within which the tumours are formed, the microenvironment determines tumour behaviour, and together they impact cancer phenotype.

3.2 Classification

Hypoxia can be broadly divided into two basic types: chronic and acute. However, there are no clear cutoff between the two types and there are typically mixed heterogeneous patterns throughout the tumour.

3.2.1 Chronic Hypoxia

Chronic, or diffusion-limited hypoxia, was first described by Thomlinson and Gray in 1955, and is caused by consumption of oxygen by cells close to the blood vessels, leaving an inadequate supply of oxygen and nutrients to the cells further away from the vessels. A distance of approximately 180 µm from vascularised stroma was shown to be the diffusion distance of soluble oxygen (Thomlinson and Gray 1955). It can also be caused by reduced oxygen content in the blood, such as in anaemia, which may be caused by tumour-related factors as well as anti-cancer therapy or carboxyhaemoglobin formation in heavy smokers, and by compromised perfusion of leaky microvessels. Chronic hypoxia usually occurs after several hours to days of hypoxia, long enough to induce changes in gene expression (Bayer et al. 2011).

3.2.2 Acute Hypoxia

Acute hypoxia, otherwise known as cycling, transient, intermittent, or perfusion-limited hypoxia, occurs because of fluctuations in the perfusion of tumour vasculature. This results in time periods of better or worse oxygenation of tumour areas. This type of hypoxia may be ischaemic due to transient occlusion of blood flow by blood clots or tumour cells, or hypoxaemic due to transient reduction in the oxygen content within microvessels (Harris 2002). Acute hypoxia may have greater effects than chronic hypoxia on the development of an invasive phenotype with hypoxia-reoxygenation episodes resulting in genomic instability, accelerated growth and metastases (Rofstad et al. 2007).

3.3 Biological Consequences

Tumour cells exposed to hypoxia activate many signalling pathways, which act in an integrated network affecting common downstream pathways. They work together to ensure adaptation to overcome the lack of oxygen and nutrients. They induce changes such as angiogenesis, glycolysis, inhibition of apoptosis and downregulation of cell adhesion molecules resulting in tumour cell detachment, all of which lead to the development and selection of more aggressive clones of tumour cells.

3.3.1 Metabolism

Glucose is the main source of energy for cells and enters the cell through a family of glucose transporters (GLUT1 to 4). Energy in the form of ATP is generated under aerobic conditions via glycolysis or the tricarboxylic (TCA) cycle. During glycolysis, glucose is metabolised to pyruvate in the cytosol to produce 2 ATPs from each molecule of glucose. Pyruvate enters the mitochondria and is oxidised by pyruvate dehydrogenase (PDH) to produce acetyl coenzyme A (CoA), which enters the TCA cycle. Acetyl CoA is metabolised through a series of reactions generating nicotinamide adenine dinucleotide hydrogen (NADH), a reducing agent used by the oxidative phosphorylation pathway in mitochondria. NADH passes electrons derived from the TCA cycle to the electron transport chain (ETC) in mitochondria, and combines with oxygen to produce water and a proton gradient that is used to generate 36 ATPs per glucose. In the mitochondria ETC, electrons transfer steps are catalysed at Complex I (NADH dehydrogenase or ubiquinone oxidoreductase) and III (coenzyme Q or cytochrome c reductase) and is transferred to O_2 at Complex IV (cytochrome c oxidase). Each step is coupled with proton translocation across the inner mitochondrial membrane, driving ATP synthesis at Complex V. Electrons can escape the ETC and be captured by O_2, forming reactive oxygen species (ROS) and resulting in oxidative stress (Sabharwal and Schumacker 2014).

Under anaerobic conditions the lack of oxygen to act as the final electron acceptor at complex IV in the ETC prevents aerobic metabolism from progressing. Pyruvate is not used in the TCA cycle, but converted to lactate in the cytosol by lactate dehydrogenase (LDH) to regenerate nictotinamide adenine dinucleotide (NAD^+) from reduced NADH, which is required by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for additional cycles of glycolysis. PDH is phosphorylated and inactivated by pyruvate dehydrogenase kinase (PDK) (Lu et al. 2002).

Cancer cells have a high rate of glucose consumption even in the presence of oxygen, known as the Warburg effect or aerobic glycolysis (Warburg et al. 1927). ATP production via glycolysis is much faster but less efficient and cancer cells avidly consume glucose to meet their increased energy and biosynthesis needs. A constant high rate of glycolysis in tumour cells uses up the NAD^+ pool and glycolysis cannot be sustained unless NAD^+ is regenerated, which is achieved by increasing lactate production.

3.3.2 Regulation of Hypoxia Driven Pathways

The underlying mechanisms that promote hypoxia and influence the level of hypoxia in tumour are currently unclear. There are two fundamentally different concepts for hypoxia driven pathways. One concept describes that hypoxia provides an environment for the selection of mutant cancer cells that tolerate hypoxic conditions (Graeber et al. 1996). Further mutations allow the cell to develop tolerance

mechanisms, giving rise to a population of cells resistant to the hypoxic microenvironment. One such mutation is the p53 gene, which has been shown to play an important role in tolerance to hypoxia; when p53 mutant cells were mixed with wild type cells and grown under hypoxic condition, only the p53 mutant cells could be selectively grown (Sermeus and Michiels 2011). The second mechanism is adaptation of normal physiological responses to hypoxia, for example by inducing factors such as hypoxia-inducible factors (HIFs) (Wouters and Koritzinsky 2008). There are a large number of oxygen sensitive enzymes and signalling pathways that are present in all cells, and hypoxia reduces the activity of enzymes that use oxygen as co-factor and leads to changes in cellular signalling.

The best understood pathway is hypoxia-inducible factor (HIF) pathway, which is stabilised due to the inhibition of the PHD (Prolyl Hydroxylase Domain oxygen sensor) enzymes (Rabinowitz 2013). The two other major cellular pathways which have been shown to be influenced by hypoxia include the kinase mammalian target of rapamycin (mTOR) pathway, which is an important moderator of metabolic signals, and the unfolded protein response (UPR), a cellular stress response related to the endoplasmic reticulum (Wouters and Koritzinsky 2008). Although they are activated independently a number of studies suggest that HIF, mTOR and UPR mediated responses to hypoxia act in an integrated fashion, both influencing each other and the common downstream pathways that affect gene expression, metabolism, cell survival, tumourigenesis, tumour growth and therapy resistance.

Below we briefly review the importance of these pathways in relation to hypoxia.

3.3.2.1 Hypoxia-Inducible Factor-1 Signalling Pathway

Tumour cell responses to hypoxia are initiated through activation of the hypoxia-inducible factor (HIF) family of transcription factors. The HIF family comprises the HIF-1α, HIF-1β, HIF-2α, and HIF-3α subunits, the most important of which is HIF-1 (Marxsen et al. 2004) HIF-1 is a heterodimeric protein consisting of an oxygen-regulated α subunit and a constitutively expressed oxygen-independent β subunit. The β subunit of HIF is a constitutive nuclear protein present in normoxic cells.

Under conditions of normal oxygen HIF-1α is rapidly degraded by the Von-Hippel-Lindau (VHL) tumour suppressor protein via the ubiquitin-proteasome pathway. The α subunits of HIF are hydroxylated at conserved proline residues P402 and P564 by propyl hydroxylase (PHD), which use oxygen as a co-substrate, resulting in recognition and ubiquitination by the VHL E3 ligase, labelling them for degradation by the proteasome. HIF-1α accumulation is also prevented under normoxia by factor inhibiting HIF-1 (FIH), which hydroxylates the transactivation domain of HIF-1α. This inhibits the binding of CREB binding protein (CBP) and p300 transcription co-factors to the HIF-1 complex (Fig. 3.1) (Semenza 2000).

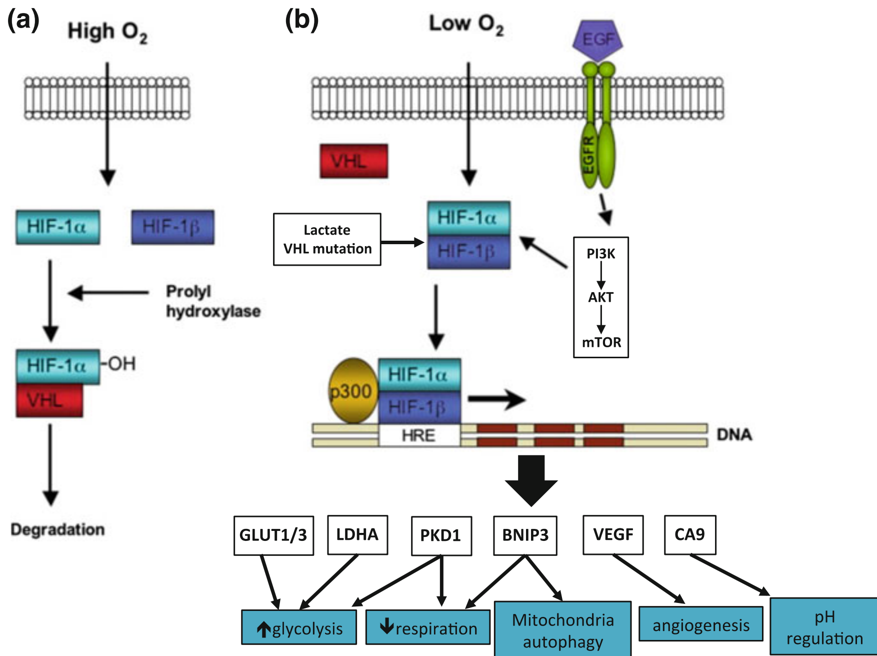

Fig. 3.1 HIF-1 activation pathway: **a** Under normoxia HIF-1α is hydroxylated by propyl hydroxylase and is degraded by VHL via the ubiquitin-proteasome pathway. **b** Under hypoxia propyl hydroxylase and FIH are inhibited and HIF-1α is stabilised, allowing heterodimerisation with HIF-1β and subsequent binding to the hypoxia-responsive elements in target genes. This results in the upregulation of proteins that promote cell survival. HIF-1α can expression can also be upregulated by EGFR and PI3 K signaling pathways. Figure adapted from Rademakers et al. (2008). Reproduced with permission from Elsevier

Hypoxia inhibits hydroxylation of HIF-1α by PHD and FIH, which results in stabilisation and accumulation of HIF-1α. HIF-1α translocates to the nucleus where it heterodimerises with constitutively expressed HIF-1β, and binds to the hypoxia-responsive elements (HREs) in the promotors of target genes. Genes that help overcome hypoxia, such as those involved in cell survival under oxidative stress, erythropoiesis, angiogenesis, glucose metabolism and pH regulation are upregulated, resulting in the upregulation of more than 100 proteins that promote survival and increased aggressiveness of hypoxic tumour cells (Semenza 2000).

Vascular endothelial growth factor (VEGF) is upregulated by HIF-1α in hypoxia and is the strongest inducer of angiogenesis, stimulating the proliferation of endothelial cells and the formation of new blood vessels. VEGF also acts as a tumour cell survival factor, inhibiting cell apoptosis by inducing the anti-apoptotic protein Bcl-2 (Ferrara et al. 2003).

Under hypoxia HIF-1α mediates a switch from oxidative to glycolytic metabolism (Lu et al. 2002). HIF-1α activation affects cellular glucose metabolism by inducing the transcription of genes involved in increased glucose uptake, such as

the glucose transporters GLUT1 and GLUT3, and stimulation of glycolytic enzymes that breakdown intracellular glucose. HIF-1α induces lactate dehydrogenase A (LDHA), increasing the conversion of pyruvate to lactate. In addition, HIF-1α activates pyruvate dehydrogenase kinase (PDK), which inactivates pyruvate dehydrogenase (PDH), actively shunting pyruvate away from the mitochondria. This reduces flux through the TCA cycle, reducing delivery of NADH to the ETC. Mitochondrial function is therefore reduced, decreasing oxygen demand in hypoxic cells. In addition HIF-1α induces BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) expression, which triggers mitochondrial autophagy, decreasing mitochondrial mass and oxygen consumption. [18]HIF-1α also diverts pyruvate to be phosphorylated by hexokinase to glucose-6-phosphate, which is used in anabolic pathways such as the pentose phosphate pathway, by upregulating hexokinase. This pathway generates the precursors of nucleotides and amino acids required for the rapid tumour cell growth and proliferation. Hypoxia also triggers the generation of reactive oxygen species (ROS) by the ETC, which is released into the cytosol where they inhibit PHDs, resulting in stabilisation of HIF-1α (Chandel et al. 2000). Glycolytic metabolism results in acidosis of the tumour microenvironment due to increased lactate and carbon dioxide levels, which are actively expelled from the tumour cells into the extracellular matrix. HIF-1α upregulates monocarboxylate transporter 4 (MCT4), which removes lactate from the cell, carbonic anhydrase 9 (CA9), which catalyses the conversion of carbon dioxide released during the pentose phosphate pathway to carbonic acid, and sodium-hydrogen exchanger (NHE1), which maintains an alkaline intracellular pH and acidic extracellular pH. Acidosis induces the secretion of matrix-degrading hyaluronidase and metalloproteinases by tumour-associated fibroblasts, creating a tumour microenvironment favourable for invasion and migration (Fang et al. 2008). In addition it reduces anti-tumour immunity, inhibiting the activity of dendritic cells and T cells, and the efficacy of drug uptake by tumours, further driving tumour progression (Balamurugan 2015).

HIF-1α activation has many other downstream effects including the expression of several proteins important in epithelial-mesenchymal transition, preparing tumour cells for invasion and migration, such as SNAIL, SLUG, TWIST (Yang et al. 2008), and inhibition of E-cadherin (Krishnamachary et al. 2006). It has also been implicated in the regulation of cancer stem cells , through activation of NOTCH1, and tumour-mediated immune suppression through attenuation of T cell function (Doedens et al. 2010). HIF-1α is also involved in the activation of tumour-associated inflammatory signalling in conjunction with nuclear factor-kappa B (NF-κB) (Bruning et al. 2012), which activates genes that promote cell proliferation and cell survival, and STAT3, which also works with HIF1α to activate VEGF (Jung et al. 2005). In addition, miRNAs are also activated, with direct binding of HIF-1α to a HRE on the proximal miR-210 promotor (Huang et al. 2009).

Factors other than hypoxia can also activate HIF-1α, such as pyruvate and lactate, indicating a positive feedback mechanism. HIF-1α expression can also be upregulated under conditions of normoxia, such as through activation of the

receptors of the tyrosine kinase family including EGFR and HER2/erbB2, activation of the PI3K-AKT-mTOR pathway, and genetic mutations including VHL inactivation, loss of PTEN, p16^{INK4a} and p53 tumour suppressors (Span and Bussink 2015).

3.3.2.2 mTOR Signalling

The adaptive response to hypoxia involves inhibition of energy-intensive cellular processes including protein translation. This effect is mediated in part through a decrease in the kinase activity of mammalian target of rapamycin complex 1 (mTORC1) (Vadysirisack and Ellisen 2012). The mTOR pathway responds to microenvironment signals such as availability of growth factors, energy (ATP) and nutrients including lipids and amino acids. These factors influence upstream kinase signalling pathways that all converge upon mTOR and the outputs of mTOR drive changes in the metabolic rate of the cells (Wouters and Koritzinsky 2008). Activation of mTOR requires oxygenation as one of its inputs and low oxygenation due to hypoxia inhibits signalling through this pathway (Magagnin et al. 2008).

Mild to moderate hypoxia levels suppress mTOR through a HIF-1 independent pathway involving the activation of tuberous sclerosis protein 1(TSC1)-TSC2 complex via adenosine monophosphate-activated protein kinase (AMPK) or via transcriptional regulation of REDD1. Hypoxia reduces the phosphorylation and activation of 4EBP1, an important negative regulator of protein synthesis, by mTOR (Magagnin et al. 2008; Rouschop et al. 2011). mTOR is a principle regulator of autophagy under nutrient depleted conditions, which is regulated by BNIP3 and AMPK during hypoxia. In addition, mTOR interacts with HIF-1α, promoting the rate of HIF-1α mRNA translation. In tumour cells hypoxia has been shown to be less effective at inhibiting mTOR through mechanisms such as constitutive mTOR activity or loss of function of TSC2 or PTEN, the negative regulator of this pathway (Wouters and Koritzinsky 2008).

The shut off of protein synthesis through mTOR inhibition is important for survival of hypoxic condition. Paradoxically mTOR is an oncogene and many known negative regulators of mTOR such as TSC1, TSC2, LKB1 and PTEN signalling have been shown to function as *bona fide* tumour suppressors, yet hypoxic cells need to shut down this pathway to survive metabolic stress.

3.3.2.3 The Unfolded Protein Response (UPR)

Messenger RNA translation, post-translational modifications and protein folding takes place in the endoplasmic reticulum (ER). The folding of these proteins in ER requires disulphide bind formation which is an oxidation reaction (Nagelkerke et al. 2013).

Under conditions of stress, such as severe hypoxia, misfolding of proteins occur which accumulate in the ER, resulting in ER stress. This activates the unfolded protein response, which aims to relieve this stress and increase cell survival by activating the ER stress sensors such as protein kinase R-like endoplasmic reticulum kinase (PERK). PERK activation results in inhibition of global mRNA translation and protein synthesis to reduce the load of misfolded proteins and lower energy demands. It also upregulates the expression of genes involved in resistance to oxidative stress and amino acid metabolism to promote cell survival, and enhances HIF-1α transcriptional activity. ER-associated degradation and autophagy occur to remove misfolded proteins, further reducing ER stress (Pereira et al. 2014). ER stress also modulates the expression of VEGF, stimulating angiogenesis and promoting cell survival of rapidly growing cells. In addition VEGF signalling can activate the UPR in endothelial cells in the absence of ER stress via mTOR signalling, further promoting angiogenesis (Wang et al. 2012). The UPR is upregulated in tumours and is an important survival response in conditions of prolonged hypoxia, increasing the threshold for apoptosis. Tumours cells with abrogated PERK activity show significantly reduced clonogenic survival and decreased ability to tolerate moderate to extreme hypoxia, with higher levels of apoptosis in hypoxic areas (Bi et al. 2005).

3.3.3 Micro RNAs

Several studies have demonstrated through genome-wide miRNA profiling that miRNA expression in tumour cells is altered in response to hypoxia (Hebert et al. 2007; Hua et al. 2006; Kulshreshtha et al. 2007; Guimbellot et al. 2009; Bruning et al. 2011; Crosby et al. 2009). However due to differences in tumour cell lines, variations in exposure to hypoxia severity and duration, as well as differences in microarray hybridisation platforms resulting in different ranges of miRNAs screened, there has been limited overlap in the patterns of miRNA up- and downregulation. Next generation sequencing techniques have revealed a greater number of previously not identified miRNAs, highlighting the complexity of miRNA response to hypoxia (Camps et al. 2014).

MiR-210, however, has been robustly and consistently induced by hypoxia, in both normal and cancer cells. It has also been shown to be overexpressed in a variety of solid tumours and patient plasma or serum, including HNSCC, where it is generally associated with poor clinical outcome (Wang et al. 2014; Gee et al. 2010). MiR-210 is maintained at low levels in normoxia. Under hypoxic conditions miR-210 is induced independently and directly by HIF-1α through interaction with its promotor sequence, and targets genes involved cell cycle regulation, mitochondrial function, apoptosis, angiogenesis and metastases. MiR-210 promotes stabilisation of HIF-1α and inhibit PHDs preventing the degradation of HIF-1α (Chang et al. 2013).

MiR-210 targets the MYC antagonist MAX network transcriptional repressor (MNT), resulting in MYC activated cell cycle progression, and the cell cycle regulator E2F transcription factor 3 (E2F3), promoting proliferation and inhibiting apoptosis (Zhang et al. 2009). Apoptosis is also inhibited by targeting apoptosis-inducing factor mitochondrion-associated 3 (AIFM3), reducing caspase-dependent apoptosis, and caspase-8-associated protein 2 (CASP8AP2), an integral protein in the cleavage and activation of apoptosis initiator caspase-8 (Kim et al. 2009). MiR-210 has been found to bind to two sites in the 3′UTR of RAD52 mRNA, resulting in degradation. RAD52 is involved in the repair of double stranded DNA breaks and therefore miR-210 suppression of RAD52 increases genetic instability (Crosby et al. 2009). MiR-210 has a repressive effect on the mitochondrial ETC, aiding the metabolic shift to glycolysis in hypoxia. It targets and suppresses several integral components of the mitochondrial ETC such as cytochrome oxidase assembly protein-10 (COX-10), succinate dehydrogenase subunit D (SDHD), compromising electron transport and promoting glycolysis (Puissegur et al. 2011). In addition miR-210 suppresses iron-sulphur cluster scaffold proteins (ISCU), which catalyse the assembly of iron-sulphur clusters that are critical for enzymes in the TCA cycle and for the function of ETC complexes I, II and III (Chan et al. 2009). Angiogenesis is stimulated by the suppression of ephrin-A3 (EFNA3), which stimulates normoxic endothelial cell to form capillary-like structures (Fasanaro et al. 2008). In addition vacuole membrane protein 1 (VMP1), which inhibits cell migration and invasion, is suppressed, inducing a more metastatic phenotype (Ying et al. 2011).

MiR-210 is also reported to have a tumour suppressor role, inducing cell cycle arrest through fibroblast growth factor receptor-like 1 (FGFRL1) (Zuo et al. 2015) and apoptosis through suppression of anti-apoptotic Bcl-2 expression (Chio et al. 2013). As hypoxia can influence both cell death and survival the role of miR-210 is likely to depend on the cell type, extent and duration of exposure to hypoxia, and reflect the adaptation of tumour cells to a hostile environment.

3.4 Clinical Consequences in HNSCC

3.4.1 Resistance to Radiotherapy

Radiosensitvity rapidly decreases when the pO_2 in a tumour is less than 25–30 mmHg (Hockel and Vaupel 2001). In 1953 Gray et al. (1953) determined the radiation dose required to achieve the same biological effect is 2.8–3 times higher in the absence of oxygen than presence. When ionising radiation is absorbed in tissue, free radicals are produced as a result of ionisations either directly in the DNA itself, or indirectly in other molecules such as water. Free radicals break chemical bonds and initiate a chain of events that results in DNA damage. Oxygen molecules are able to react with the free radicals and create a stable change in the chemical composition of the DNA damage. Oxygen therefore enhances the damage by

"fixing" or making the damage permanent. In the absence of oxygen the target is chemically restored and the damage is repaired. In order for oxygen to consolidate radiation-induced damage it needs to be present at the time of irradiation or within a few milliseconds (Howard-Flanders and Moore 1958). Other hypoxia-induced mechanisms also contribute to radioresistance including glycolytic metabolism and lactate accumulation, which act as antioxidants and scavenge free radicals (Brizel et al. 2001; Sattler et al. 2010).

The changes in hypoxia heterogeneity during a course of fractionated radiotherapy (RT) can affect the response. During the early stages of treatment cell density decreases as the well-oxygenated tumour cells are killed. This results in reduced intratumoural pressure and increased vascular density leading to tumour reoxygenation (Bussink et al. 2000). This reoxygenation does not increase HIF-1α degradation, but the increase in ROS formation stabilises HIF-1α and increases the expression in a hypoxia-independent manner (Harada et al. 2009). This, together with accelerated repopulation that occurs at the later stages of RT, means that the entire cumulative dose of RT needs to be delivered.

3.4.2 Resistance to Chemotherapy

Some chemotherapeutic agents, such as etoposide, are dependent on the presence of oxygen for maximum cytotoxic effect (Wozniak et al. 1984). The blood vessels in hypoxic tumours are disordered and leaky, preventing efficient delivery of chemotherapy drugs. Hypoxic cells are distant from blood vessels and therefore not adequately exposed to drugs (Tannock 1998). Alkylating agents, such as cisplatin, and anti-metabolites, such as 5-FU, act during DNA synthesis by damaging DNA and initiating apoptosis. Cellular proliferation and DNA synthesis decreases as a function of distance from blood vessels and hypoxia selects for cells that have lost sensitivity to p53 mediated apoptosis, reducing the cytotoxic effect. Hypoxia upregulates genes involved in drug resistance, such as multidrug resistant 1 (MDR1) leading to the increased expression of P-glycoprotein, which is associated with tumour resistance to chemotherapeutics (Comerford et al. 2002). The acidic tumour microenvironment creates a pH gradient between the tumour cell and extracellular space, inhibiting the accumulation of drugs that are weak bases, such as doxorubicin (Skovsgaard 1977).

3.5 Therapeutic Approaches to Overcome Hypoxia Radioresistance in HNSCC

Hypoxia represents a potential target for therapy. Various strategies have been used to overcome hypoxia in HNSCC, summarised in Table 3.1. A meta-analysis of 32 clinical trials of various strategies of hypoxic modification demonstrated a small but

Table 3.1 Summary of methods investigated to overcome hypoxic radioresistance

Method	Example
Increasing oxygen availability	Hyperbaric oxygen ARCON Blood transfusion, erythropoeitn
Radiosensitisers	Nimorazole
Hypoxia cytotoxin	Tirapazamine
Increasing radiotherapy dose	Dose painting
Other	Anti-angiogenic agents with RT HIF-1 targeting

significant improvement in locoregional control (LRC), disease specific control (DSS) and overall survival (OS) in HNSCC treated with primary RT (Overgaard 2011). Hypoxia stratification is required to identify patients at risk of treatment failure but also patients with normoxic tumours, who do not gain benefit from additional treatment, and can avoid additional toxicity.

3.5.1 Improving Haemoglobin (Hb) Levels

Many studies have demonstrated that low haemoglobin level prior to treatment associated with significant reduction in survival and increase in locoregional failure in HNSCC after RT or chemoradiation (Fortin et al. 2008; Lee et al. 1998). Over 1000 patients pooled from two studies demonstrated that high Hb was significantly associated with higher OS and the administration of blood during therapy increased Hb level. However blood transfusions had no impact on LRC, DSS or OS (Hoff et al. 2011). Exogenous erythropoietin administration to stimulate red blood cell production and correct anaemia has been investigated. However this did not improve tumour control or survival as demonstrated in 2 meta-analyses, but suggested an increase in mortality (Bohlius et al. 2009; Lambin et al. 2009).

3.5.2 Increasing Oxygen Availability

Hyperbaric oxygen is the administration of 100% oxygen at higher than normal atmospheric pressure. This elevated pressure results in increased pO_2 in tissues and has been delivered in hyperbaric oxygen chambers with RT. This strategy has been shown to significantly improve tumour control and mortality, but also causes an increased rate of severe radiation tissue injury and chance of oxygen toxic seizures during therapy (Bennett et al. 2008).

Carbogen (95% oxygen and 5% carbon dioxide) breathing before RT did not improve tumour control (rubin 1979 carbogen), but combined with accelerated radiotherapy and nicotinamide (ARCON, accelerated radiotherapy with carbogen

and nicotinamide) has been shown to result in high LRC in advanced larynx and oropharynx SCC (Kaanders et al. 2002). This combination aims to limit clonogenic repopulation during therapy by reducing the overall duration of RT by delivering more than one fraction a day, decrease diffusion-limited hypoxia with inhalation of carbogen, and decrease perfusion-limited hypoxia by administering nicotinamide, a vasoactive vitamin B_6 analogue. A more recent study compared accelerated RT with ARCON in larynx SCC and did not demonstrate a benefit. However, a subgroup of patients had their hypoxic status assessed with pimonidazole IHC, which stains regions of hypoxia, and significant improvement in regional control and disease free survival (DFS) was found in the high pimonidazole staining group treated with ARCON (Janssens et al. 2012).

3.5.3 Radiosensitisers

Cellular sensitivity to RT can be improved by increasing the apparent cellular pO_2 levels using radiosensitisers. Nitromidazoles are chemical compounds that mimic the radiosensitising effect of oxygen by inducing and stabilsing free-radical mediated double-stranded DNA breaks. The benefit of the addition of a nitroimidazole compound to RT was carried out in many studies, without clinical benefit (Lee et al. 1995; Van den Bogaert et al. 1995). However these studies used the older generation of the drug which is limited by neurotoxicity. Overgaard (1994) carried out a meta-analysis of over 7000 patients treated with nitroimidazole-based radiosensitisers in various tumour types of unknown hypoxia status and found an improvement in LRC and OS, with the greatest benefit in HNSCC. This was followed by the DAHANCA 5 study which randomized over 400 HNSCC patients to receive RT with nimorazole or RT with placebo. LRC and DSS were significantly higher with the addition of nimorazole (Overgaard et al. 1998). A phase II study investigated accelerated RT with nimorazole and cisplatin in 227 patients with locally advanced HNSCC and recently reported a high 5-year OS of 75% (Bentzen et al. 2015).

3.5.4 Hypoxic Cytotoxins

This group of drugs, of which tirapazamine is the most widely investigated, have a direct cytotoxic effect on hypoxic cells. Tirapazamine (TPZ) is a prodrug that requires one-electron reduction to a radical by intracellular reductases. In normoxia the unpaired electron in the radical is rapidly transferred to molecular oxygen, forming superoxide and regenerating the initial prodrug. In hypoxic conditions the radical accumulates and induces DNA damage, in part due to poisoning of topoisomerase II (Brown 1993).

TPZ combined with cisplatin and RT in HNSCC has shown to be well tolerated in phase I and II studies (Lee et al. 1998; Rischin et al. 2001). Rischin et al. carried out a phase II randomised trial of this combination versus cisplatin and RT and demonstrated a trend towards improved 3-year survival with acceptable toxicity (Rischin et al. 2005). However, further studies have not shown a benefit in survival or quality of life from the addition of TPZ (Rischin et al. 2010). Rischin et al. also carried out a substudy of 45 patients who had their tumour hypoxic status assessed by ^{18}F-fluoromisonidazole, a hypoxic radiotracer, PET scans. Hypoxic patients performed poorly in the absence of TPZ augmentation, whereas the rate of complete response in the hypoxic group receiving TPZ was nearly as high as the rate for normoxicchemoRT patient. Patients with hypoxic tumours were more likely to develop distant metastatic disease compared with normoxic patients (Rischin et al. 2006).

3.5.5 Hypoxia Image-Guided Radiotherapy

Intensity modulated radiotherapy (IMRT) is used in the treatment of HNSCC. The intensity of the radiation beam can be modulated to reduce doses to normal structures without compromising the doses to the tumour. Tumour recurrence after compete response to radiotherapy has been shown to occur predominantly in high radiation dose regions, implying that tumours have radioresistant subvolumes, potentially due to hypoxia, within this region (Hendrickson et al. 2011). IMRT dose painting is a method of assigning different dose levels to structures within the same treatment fraction, resulting in the potential for higher total doses to selected targets (Galvin and De Neve 2007). Dose painting requires functional imaging such as positron emission tomography to direct regions for dose escalation. Intensification of intratumoural subvolumes of hypoxia has been investigated as a strategy to improve outcome in RT planning studies and radiobiological modelling studies only as this requires accurate and stable identification of hypoxia to guide target volume delineation.

3.5.6 Targeted Therapies

3.5.6.1 Targeting Angiogenesis: VEGF Receptors

High VEGF expression levels has been associated with worse overall survival in HNSCC (Kyzas et al. 2005). As tumour vasculature is abnormal, increased VEGF expression and microvascular density does not lead to increased blood flow and oxygen delivery. Targeting VEGF is a strategy to improve oxygenation by normalising the tumour vasculature and thereby increase response to RT. RT can lead

to stimulation of angiogenesis through upregulation of VEGR and therefore anti-angiogenic therapy has been tested with RT (Koukourakis et al. 2001). Bevacizumab is a humanised monoclonal antibody against VEGF approved for use in colorectal cancer, and has been investigated in phase II studies concomitant with chemoRT in HNSCC with encouraging results (Fury et al. 2012). It inhibits endothelial cell growth and function, disrupting the formation of capillary networks, and has been shown to enhance RT response in HNSCC xenograft models (Hoang et al. 2012). Anti-angiogenic agents can improve tumour oxygenation by reducing the number of oxygen consuming endothelial and tumour cells, and the number of inefficient blood vessels. This also results in less leaky vessels and decreased interstitial pressure, improving perfusion (Willett et al. 2004).

3.5.6.2 Targeting HIF-1

Targeting HIF-1 is an attractive therapeutic strategy to overcome the effects of hypoxia. Drugs that inhibit HIF-1 activity are already in clinical use, such as the topoisomerase I inhibitor topotecan and mTOR inhibitors which decrease HIF-1 synthesis, the anthracycline doxorubicin which suppresses HIF-1 DNA binding, and the proteasome inhibitor bortezomib, which reduces HIF-1 transactivation (Semenza 2010). Newer drugs have been developed, such as PX-478 that inhibits HIF-1 by inhibiting HIF-1 mRNA translation and HIF-1 dequbiquitination and is being investigated as a phase I trial. In HNSCC tumour models this drug provided significant radiosensitisation of hypoxic cell lines and xenografttumours (Schwartz et al. 2009).

3.6 Methods to Detect Hypoxia in HNSCC

Accurately detecting and quantifying hypoxic tumours is essential in identifying patients who have aggressive, treatment resistant disease and has been one of the limiting factors for translating hypoxia-modification strategies into routine practice.

3.6.1 Clinical Studies Using Eppendorf polarographic Needle Electrodes

Approximately 50–60% of human tumour contain hypoxic and/or anoxic tissue regions that are heterogeneously distributed within the tumour (Vaupel et al. 2007). Many clinical studies, mainly in HNSCC and cervix cancers, using Eppendorf polarographic needle electrode histography have directly demonstrated that tumour

hypoxia predicts for decreased local control, increased disease recurrence and reduced overall survival. This technique involves inserting an electrode into multiple sites in a tumour and measuring the pO_2 at several points per needle track and data from all tracks form a histogram. In head and neck cancers the pO_2 measurements between the primary tumour and metastatic lymph nodes have shown little difference, and nodal measurements have been used to represent the hypoxic status of the patient (Becker et al. 1998).

In head and neck cancer, Gatenby et al. (1988) in 1988 measured the oxygen tension in 31 lymph node metastases from HNSCC patients, and demonstrated a significant relationship between low mean pO_2 measurements and poor response to radiotherapy. The volume of tumour containing low oxygen levels was also found to be important. Nordsmark et al. (1996) measured the pretreatment oxygenation status initially in 34 lymph nodes and 1 primary using the Eppendorf polarographic needle electrode histography and evaluated the tumour oxygenation status as the percentage of pO_2 values ≤ 2.5 mmHg (hypoxic fraction 2.5, HF2.5). The median HF2.5 of 15% was used to define hypoxia and these patients had significantly poorer locoregional control (LRC). This was confirmed by the same group in a further 35 patients (Nordsmark and Overgaard 2000). Brizel et al. (1997, 1999) assessed 63 patients for pretreatment tumour oxygenation using the primary site or metastatic lymph node. A preliminary study had shown significantly worse disease free survival (DFS) at 12 months for patients with a median $pO_2 < 10$ mmHg, and this was used to define hypoxic tumours. Whether the measurement was taken at the primary or nodal site did not affect the DFS and hypoxic tumours had significantly worse 3 year LRC, DFS and overall survival (OS), independent of tumour stage. However, a small study of 25 patients did not find an association between pretreatment oxygen levels and LRC or OS (Adam et al. 1999).

Stadler et al. (1999) identified the importance of the hypoxic subvolume, defined as the percentage of pO_2 values <5 mmHg multiplied by the total tumour volume. Fifty-nine HNSCC primaries and nodes were assessed and the median pO_2 was 16 mmHg, with no difference between the sites. On multivariate analysis the hypoxic subvolume and the HF5 were significant prognostic factors for survival. This was confirmed in a further 125 patients, where hypoxic tumour volume was a strong and independent prognostic factor for survival (Dunst et al. 2003). Rudat et al. (2000) evaluated the repeatability and predictive value of pO_2 Eppendorph electrode histography in HNSCC. High variability was seen in patients who had two repeated independent measurement of the same tumour. In 41 patients with follow up data, locally advanced HNSCC the fraction of $pO_2 \leq 2.5$ mmHg was a significant prognostic factor for survival.

A joint analysis of prospectively collected Eppendorf pO_2 measurements from multiple centres was performed, which consisted of 397 patients with HNSCC (Nordsmark et al. 2005). Median tumour pO_2 was 9 mmHg and multivariate analysis demonstrated that HP2.5 greater than population median (19%) was associated with poorer OS at 3 years, providing strong evidence that tumour hypoxia has a significant role in HNSCC. Table 3.2 summaries the clinical data from HNSCC.

Table 3.2 Summary of Eppendorf electrode histography studies in HNSCC

No. of patients	Outcome	Ref.
31	Mean pO_2 20.6 mmHg in complete responders versus 4.7 mmHg in non-responders to radiotherapy	Gatenby et al. (1988)
35	Median pO_2 14 mmHg Median HF2.5 > median (15%) significantly worse LRC	Nordsmark et al. (1996)
31	Median pO_2 12 mmHg, median HF2.5 30% Median HF2.5 > median (15%) significantly worse LRC	Nordsmark and Overgaard (2000)
63	Median pO_2 4.8 mmHg for 24 primary sites Median pO_2 4.3 mmHg for 39 nodes Median pO_2 < 10 mmHg significantly worse LRC, DFS, OS	Brizel et al. (1999, 1997)
41	Median pO_2 10 mmHg HF2.5 > median (21%) worse OS	Rudat et al. (2000)
397	Joint analysis of prospectively collected data in HNSCC Median tumour pO_2 9 mmHg HP2.5 > median (19%) associated with poorer OS	Nordsmark et al. (2005)
25	Mean pO_2 20.2 mmHg No prognostic impact on outcome	Adam et al. (1999)
59	Median pO_2 16 mmHg Hypoxic subvolume > 6 cm3, HF5 > median (30%) worse overall survival	Stadler et al. (1999)
125	Median pO_2 9.4 mmHg Hypoxic volume prognostic factor for OS	Dunst et al. (2003)

Table 3.3 Summary of methods used to detect hypoxia

Method	Example
Direct detection	Eppendorf oxygen electrode histography
Exogenous markers	2-nitroimidazole—pimonidazole, EF5
Endogenous markers	
Tumour protein levels Circulating markers Gene expression signatures	HIF-α1, CA9, osteopontin Osteopontin, miR-210 Hypoxia metagene, 15-gene classifier
Non-invasive imaging	DCE MRI, BOLD MRI PET—^{18}F-FMISO, ^{64}Cu-ATSM

The use of Eppendorf oxygen electrode histography has been crucial to demonstrate the adverse effects of hypoxia on outcome in the clinical setting. It has the advantage of directly measuring absolute pO_2 values and has prognostic potential. However, it has not been incorporated into routine clinical practice as it is an invasive procedure restricted to accessible tumours. It also requires an experiences operator but still exhibits large inter-observer variability (Nozue et al. 1997), and only provides information on the areas sampled. In addition it cannot differentiate between tumour and normal tissues (Vaupel et al. 2007). Other methods that have been investigated are summarised in Table 3.3.

3.6.2 Exogenous Markers of Hypoxia

The most commonly used exogenous markers of hypoxia are 2-nitroimidazole compounds such as pimonidazole and pentafluorinatedetanidazole (EF5) (Evans et al. 2000; Raleigh et al. 1998). They are irreversibly bioreduced by cellular nitroreductases and bind to thiol-containing proteins in viable hypoxic cells, forming stable adducts (Varghese et al. 1976). These adducts can then be detected by IHC staining with specific antibodies. IHC staining can quantify hypoxia by visually or digitally estimating the fraction of stained cells and provides hypoxic measurements with high spatial resolution. Binding is commonly seen adjacent to regions of necrosis and at a distance from blood vessels, in keeping with diffusion-limited hypoxia (Evans et al. 2000).

2-nitroimidazoles bind to tissue at a pO_2 level of <10 mmHg and are more sensitive at lower pO_2 levels than microelectrodes (Raleigh et al. 1999). The amount of bound marker is dependent on oxygen, the accumulation rate of individual hypoxic cells and intact nitroreductase enzymes in viable hypoxic cells (Ljungkvist et al. 2007). Differences in the activity of the enzymes can affect level detected and the accumulation rate of pimonidazole is dependent on pH (Dennis et al. 1985). The main disadvantage to exogenous markers of hypoxia is that they have to be administered intravenously prior to tissue biopsy or surgical resection, to allow fixation of the adducts that form in hypoxic regions. Pimonidazole binding has shown good correlation with Eppendorf electrode measurements in mouse models (Raleigh et al. 1999), but not in the clinical setting in cervix cancers (Nordsmark et al. 2003). Neither pimonidazole nor Eppendorf electrode pO_2 were not prognostic for outcome in 127 cervix cancer patients (Nordsmark et al. 2006).

The prognostic value of 2-nitroimidazoles has been shown in HNSCC. High pimonidazole binding in 43 biopsies was significantly associated with LRC and DFS at 2 years. This association was lost in the subgroup treated with ARCON, suggesting a predictive role for pimonidazole (Kaanders et al. 2002). Pretreatment hypoxia using EF5 binding was investigated in 22 HNSCC patients and severe hypoxia, defined as maximum 30% EF binding which approximated to a pO_2 of 0.76 mmHg, was associated with shorter event free survival at 3 years (Evans et al. 2007).

3.6.3 Endogenous Markers of Hypoxia

Endogenous markers are genes or gene products that are specifically upregulated under hypoxic conditions and can be measured on tumour specimens. HIF-1α, GLUT1 and 3, CA9 and osteopontin are detected by antibody staining and immunohistochemistry and can be assessed on formalin fixed paraffin embedded (FFPE) archival material, allowing correlation with outcomes. However the techniques of tissue processing and staining analysis have not been validated between

different laboratories, limiting comparisons and clinical applications. Samples are usually from small biopsy specimens and therefore not representative of the hypoxia heterogeneity of entire tumour. In addition their expression may be affected by the type of hypoxia, for example HIF-1α is induced and repressed rapidly and reflect acute changes in hypoxia, whereas CA9 is slow to accumulate (Sobhanifar et al. 2005). In general, studies using these markers show conflicting results, and the association between these markers and oxygen status of tissue is weak.

3.6.3.1 HIF-1α

HIF-1α is overexpressed in a wide variety of tumours. In HNSCC many studies have demonstrated an association with poor survival and poor response to chemoRT (Aebersold et al. 2001; Koukourakis et al. 2002), and has been found to be an independent prognostic factor in HNSCC. However there are also studies that show no association with prognosis (Kyzas et al. 2005; Wachters et al. 2013) and in one study of in surgically resected cohort HNSCC, HIF-1α expression was associated with significantly better DFS and OS (Beasley et al. 2002).

3.6.3.2 Glucose Transporters (GLUT)

These glucose transporters are present in normal tissue but upregulated in tumours due to increased glucose requirements in anaerobic glycolysis (Rademakers et al. 2011). In HNSCC GLUT-1 has been associated with poor treatment outcome in multiple retrospective series (Mineta et al. 2002; Oliver et al. 2004). In 58 patients treated with ARCON GLUT-1 was associated with an increased rate of distant metastasis and worse OS but GLUT-3 was associated with better LRC (Jonathan et al. 2006). Co-expression of HIF-1α and GLTU-1 significantly correlated with an increased risk of tumour-related death (Eckert et al. 2011).

3.6.3.3 Carbonic Anhydrase 9 (CA9)

CA9 is a transmembrane enzyme and a downstream target of HIF-1. It catalyses the reversible hydration of carbon dioxide to carbonic acid and is involved in pH regulation. Elevated expression has been demonstrated at $pO_2 < 20$ mmHg (Wykoff et al. 2000). Overexpression of CA9 has been found in different types of cancer including HNSCC. Kaanders et al. (2002) showed that the expression of CA9 in HNSCC demonstrated some overlap in the distribution pattern as pimonidazole, with increasing signal at greater distance from blood vessels, but

overall the correlation was found to be weak and not associate with outcome. Greater CA9 expression was seen at shorter distances from the vessels, suggesting that upregulation may occur at pO_2 levels higher than that required for pimonidazole (Kaanders et al. 2002). CA9 shows conflicting results as a prognostic marker. Strong CA9 expression was found to be related to poor complete response rate after treatment with chemoRT (Koukourakis et al. 2001), but positivity was associated with better LRC and freedom from distant metastases in 58 patients treated with ARCON (Jonathan et al. 2006), or no association with LRC (Nordsmark et al. 2007). In the DAHANCA 5 study patients 320 samples were available for CA9 staining and the expression of CA9 did not correlate with any of the tumour or patient characteristics, was not a prognostic marker and did not correlate with nimorazole treatment (Eriksen et al. 2007).

3.6.3.4 Osteopontin (OPN) in Tissue and Plasma

Osteopontin (OPN) is an extracellular matrix-associated integrin-binding glycoprotein protein induced by hypoxia, initially identified in non-collagenous bone matrix. Binding of OPN to cell surface receptors on tumour cells activates integrins and matrix metalloprotein signalling pathways, increasing the risk of tumour invasion and migration (Chien et al. 2009). It is also involved in angiogenesis, promotes cell survival through PI3K/AKT and JAK/STAS3 signalling and regulation of NF-κB. It is upregulated through AKT activation, independent of HIF-1 under hypoxia (Ahmed et al. 2011). High OPN levels in both tissue and plasma have been correlated with low tumour pO_2 in HNSCC and outcome (Nordsmark et al. 2007). A retrospective study of the DAHANCA 5 trial patients demonstrated that high OPN plasma levels was associated with higher locoregional tumour failure and disease specific mortality (Overgaard et al. 2005). Patients with high OPN levels who received nimorazole had better outcomes compared with patients with high OPN levels who did not receive nimorazole. No effect was seen in the low/intermediate OPN group, suggesting that high concentrations of OPN could predict clinically relevant hypoxia and identify patients who may benefit from hypoxic modification. Lim et al. (2012) investigated the predictive potential of OPN in patients who received tirapazamine but did not find that high levels were associated with poor prognosis and found no interaction between OPN and treatment. The difference in results compared with the DAHANCA study may be due to differences in treatment protocols, but also lack of standardisation for OPN thresholds between the studies.

3.6.3.5 Gene Expression Signatures of Hypoxia

As hypoxia is influenced many biological pathways, a single marker is incapable of adequately describing this complex heterogeneous response. To improve hypoxia

specificity combining several markers in gene expression signatures of hypoxia have been investigated. Different methods have been used to derive clinically applicable hypoxic gene signatures and are summarised in Table 3.4.

In vitro derived gene sets have been described by culturing tumour cell lines under normoxic and hypoxic conditions, then assessing the differences in gene expression. Koong et al. (2000) used FaDu HNSCC and SiHa cervical SCC cell lines to identify 9 genes exhibiting greater than 3-fold induction under hypoxic conditions when using the expression of VEGF as a cut off point for assessing hypoxia-induced genes. An 84 upregulated gene signature was generated by culturing normal and transformed keratinocytes at various different oxygen levels, which could be grouped into 6 functional groups, including as metabolism/transport, angiogenesis, tissue remodelling, apoptosis, proliferation/differentiation and gene expression (Denko et al. 2003).

Chi et al. (2006) evaluated the gene expression in response to hypoxia in several primary cell lines in vitro using cDNA microarrays and found a wide variation in the response between cell and tissue types. However, a 253 gene signature was identified which were concordant with gene expression data from a distinct subset of renal tumours, allowing classification by hierarchical clustering into 2 groups with high or low expression of the hypoxia response genes. The high expression group consisted of clear cell renal carcinomas, which typically have loss of function al VHL proteins, and the low group were other histological subtypes or normal samples. Similar classification grouped breast cancers into ductal adenocarcinomas (high expression) and fibroadenomas or normal samples (low expression). In addition, the gene signature was a strong predictor of clinical outcome in independent breast and ovarian cancer datasets.

Human mammary epithelial cells were culture under hypoxic conditions at early and late time points to assess the time dependency of hypoxia-regulated gene expression (Seigneuric et al. 2007). The early response gene signature was characterised by genes related to growth, apoptosis, insulin and oestrogen receptor signalling, whereas the late response was characterised by genes involved in angiogenesis, glucose transport, proliferation, metastasis and apoptosis, and were similar to the genes identified by Chi et al. The early but not late signature was prognostic in univariate analysis, but not maintained in multivariate analysis in a breast cancer dataset. Sorensen et al. (2010) exposed 4 head and neck cell lines to different oxygen concentrations at normal or low pH, and analysed the gene expression analysed to identify genes upregulated by hypoxia, independent of pH. Hypoxia induced pH independent genes were selected if they were common in 3 of the 4 head and neck cell lines, resulting in a 27 gene signature.

Winter et al. (2007) developed a hypoxia metagene from 59 HNSCC fresh frozen samples by clustering around the mRNA expression of 10 well-known hypoxia-regulated genes, such as CA9, GLUT1 and VEGF. Strongly correlated upregulated genes appearing in >50% of clusters defined a signature comprising of 99 genes, which was found to be an independent prognostic factor for RFS in an independent head and neck cancer and breast cancer dataset. The same group derived a common hypoxia metagene consisting of 51 genes by selecting genes that

Table 3.4 Summary of hypoxia gene expression signatures

Source of signature	No. of genes in signature	Prognostic validation	Ref.
FaDu, SiHa cell lines in vitro under hypoxia and normoxia	9	No	Koong et al. (2000)
Normal cervical and dermal keratinocytes, normal stromal fibroblasts, SiHa, C33a, FaDu cells	84	No	Denko et al. (2003)
Mammary and renal tubular epithelial cell lines under hypoxia or normoxia for 12 h	253	2 breast cancer, 1 ovarian cancer datasets—prognostic for OS and RFS on univariate analysis; breast dataset—prognostic for OS on multivariate analysis	Chi et al. (2006)
Mammary epithelial cell line under hypoxia; early (1–6 h) or late (12–24 h)	Early 15 Late 93	Early signature prognostic for DSS in breast cancer dataset on univariate analysis only; late signature not prognostic	Seigneuric et al. (2007)
59 HNSCC biopsy specimens Clustering around known hypoxia regulated genes	99 hypoxia metagene	Independent prognostic factor in head and neck for RFS and breast for OS and metastasis free survival	Winter et al. (2007)
In silico generation of hypoxia co-expression networks using 3 head and neck and 5 breast cancer studies	51 common hypoxia metagene	Prognostic in breast, 2 lung, head and neck datasets	Buffa et al. (2010)
FaDu, UTSCC5, UTSCC 14, UTSCC15 cell lines under hypoxia and either normal or acidic pH	27	No	Sorensen et al. (2010)
UTSCC33, FaDu and SiHatumourxenografts—hypoxic regions identified using FAZA hypoxia tracer; 58 HNSCC fresh frozen biopsies with oxygen electrode measurements	15	FFPE samples from DAHANCA5 trial—independent prognostic factor; more hypoxic tumours treated with nimorazole had reduced incidence of locoregional failure	Toustrup et al. (2011)
DU145, HT29, MCF7 cell lines exposed to normoxia and different rimes of severe hypoxia	7 temporal and 2 general signatures	Not prognostic in breast cancer dataset	Starmans et al. (2012)

were consistently co-expressed with previously validated hypoxia-regulated genes (Buffa et al. 2010). By applying more training sets and co-expression networks a reduced metagene had prognostic significance in 4 independent datasets of breast, lung and head and neck cancers, and outperformed larger published signatures. The top 26 genes from this signature were retrospectively assessed in FFPE samples from laryngeal and bladder cancer patients treated with ARCON or CON (carbogen and nicotinamide) respectively (Eustace et al. 2013). Tumours were categorised into high and low hypoxia groups, and laryngeal tumours in the high hypoxia group showed greater benefit from ARCON than the low hypoxia group. The hypoxia signature did not predict benefit from CON in bladder cancer.

In contrast to previous studies on hypoxia gene expression, Toustrup et al. (2011) developed a classifier based on the hypoxic status of tumours. The hypoxia induced pH independent gene profile developed by Sorensen et al. was validated in vivo in a xenograft study using ^{18}F-FAZA as an exogenous hypoxia radiotracer in autoradiographic studies. Hypoxic, non-hypoxic and mixed heterogeneous tumour areas defined by ^{8}F-FAZA positive and negative regions were demarcated and dissected. All the genes investigated were significantly upregulated in hypoxic tumour areas compared with non-hypoxic areas, and all but 3 were upregulated in samples from mixed heterogenous versus non-hypoxic areas. To identify the most informative genes, a training set of 58 hypoxia-evaluated HNSCC FFPE biopsies were analysed for gene expression. The oxygenation status of these tumours had previously been evaluated in accordance to the relative number of oxygen electrode measurements less than 2.5 mmHg in their metastatic cervical lymph nodes. A 15-gene expression classifier was generated containing 15 of the in vitro identified hypoxia-responsive genes, which could best discriminate between 'more' and 'less' hypoxic human HNSCCs. This was evaluated in an independent data set, where patients with HNSCC were randomised to receive either hypoxic modification with nimorazole or placebo concomitant with radiotherapy. Patients with 'more' hypoxic tumours defined by the classifier had significantly higher cumulative incidence of locoregional failure at 5 years compared with those with less hypoxic tumours. Within the 'more' hypoxic group, patients treated with nimorazole had significantly reduced incidence of failure compared with placebo, whereas in the 'less' hypoxic group there was no significant difference in outcome, suggesting the classifier potentially has predictive value. Further subgroup analysis demonstrated that this benefit was only found in HPV-negative 'more' hypoxic tumours and not HPV-positive, whose outcome was unaffected by hypoxic modification (Toustrup et al. 2012). The same frequencies of 'more' and 'less' hypoxic tumours were in HPV-negative and positive groups as assessed by the classifier. More recently the classifier was used to assess 55 patients recruited in a randomised trial accelerated RT with or without nimorazole (Hassan Metwally et al. 2015). RT alone in 16 patients with 'more' hypoxic tumours was associated with higher locoregionaltumour failure.

More recently further investigation into the time dependent gene expression changes in response to hypoxia was carried out using prostate, colon and breast cancer cell lines exposed to hypoxia at 8 different time points between 0 and 24 h

(Starmans et al. 2012). Seven different signatures consisting of induced genes with distinct temporal profiles and 2 general hypoxia signatures were generated, but none were prognostic in a large breast cancer cohort. In contrast previously published in vivo derived signatures showed clear prognostic power, suggesting the importance of the tumour microenvironment in the response to hypoxia.

Classification of hypoxia gene expression has the potential to represent prognostic and predictive markers in cancer and has been demonstrated retrospectively to be applicable to unselected FFPE biopsy samples, and therefore translatable into clinical practice. However, the 2 smaller gene signatures (15 and 26 gene signatures) which have demonstrated predictive value, have only 4 genes that overlap, suggesting that this method alone may not identify all hypoxic tumours. Prospective validation in clinical studies is required before further conclusions can be made.

3.6.4 Magnetic Resonance Imaging (MRI)

3.6.4.1 Dynamic Contrast-Enhanced MRI (DCE MRI)

In this technique fast repeated images are acquired before, during and after the rapid administration of a small hydrophilic gadolinium-based contrast agent, which diffuses through blood vessel walls and distributes into the extracellular space. The change in signal intensity reflects tumour perfusion, vessel permeability and the volume of extracellular space (Turkbey et al. 2010). It does not correlate directly with tissue pO_2 levels but a small study in HNSCC found positive correlation between imaging parameters of poor perfusion and pimonidazole staining (Newbold et al. 2009), and with hypoxia defined by ^{18}F-fluoromisonidazole PET uptake in nodal metastases (Jansen et al. 2010).

3.6.4.2 Blood Oxygen Level-Dependent MRI (BOLD MRI)

This is an indirect measure of visualising pO_2 in blood vessels and surrounding tissue based on the paramagnetic properties of deoxyhaemoglobin, which is related to tissue oxygenation. It does not provide quantitative information on oxygen concentration and the signal can be influenced by many factors such as blood flow and pH. However, studies have shown correlation between this technique and polargraphic electrode pO_2 measurements and pimonidazole staining in prostate cancer, suggesting BOLD MRI may provide complementary information related to tissue oxygenation (Chopra et al. 2009).

3.6.5 Positron Emission Tomography (PET)

PET is a molecular imaging technique that can visualise and quantify tumours and their microenvironment. Different biological tumour characteristics can be imaged depending on the radiotracer used. Several hypoxia-specific PET radiotracers have been developed and extensively investigated as it is a potentially useful non-invasive technique for identification, quantification of hypoxia and repeated measurements after intervention. The metabolic activity demonstrated by the commonly used radiotracer ^{18}Fluorine-fluororodexoxyglucose (^{18}F-FDG) is indirectly related to the proliferative activity and oxygenation status of tumours, but cannot reliably distinguish hypoxic tumours. A good hypoxia tracer should be able to detect pO_2 levels that are clinically relevant to therapy, and be able to distinguish between normoxia, hypoxia, anoxia and necrosis. The molecule should be small and lipophilic making it highly membrane permeable leading to rapid uptake into cells and rapid clearance from normoxic cells to allow a high tumour to background contrast. It should also have a good dosimetry profile with simple radiolabelling and production, and low radiation dose to the patient. Two main classes of hypoxia imaging radiotracers are in available in the clinical trial setting: one based on the nitroimidazole compounds and one based on a complex of copper with diacetyl-bis (N^4-methylsemicarbazone) (ATSM) ligands. A meta-analysis of published hypoxia imaging studies, which included PET and other imaging modalities of hypoxia, showed a uniform tendency for poor response to radiotherapy in tumours showing a higher uptake despite the heterogeneity of the image acquisition, data analysis and treatments (Horsman et al. 2012).

3.6.5.1 F-Fluoromisonidazole (^{18}F-FMISO)

^{18}F-FMISO is a derivative of the nitroimidazole group of compounds and is the most widely studied hypoxic radiotracer. It is moderately lipophilic and enters cells by passive diffusion across the cell membrane. The nitro group of the imidazole ring structure ($R\text{-}NO_2$) is reduced by intracellular reductases to $R\text{-}NO_2^-$, which is reoxidised in the presence of oxygen and the tracer can flow back into the extracellular compartment. In conditions of hypoxia $R\text{-}NO_2^-$ can be further reduced with progressive production of $R\text{-}NH_2$ compounds that bind covalently to intracellular molecules and the tracer becomes trapped. This occurs at $pO_2 < 10$ mmHg and therefore ^{18}F-FMISO detects clinically relevant hypoxia. The process requires viable cells with functional nitroreductases and the tracer does not accumulate in necrotic cells (Prekeges et al. 1991).

3.6.5.2 Clinical Studies of ^{18}F-FMISO in HNSCC

Rajendran et al. (2006) reported the largest study ^{18}F-FMISO, which involved 73 patients with HNSCC. Uptake was seen in 85% of patients and tumour to blood ratio and hypoxic volume showed a trend to be an independent prognostic measure. Rischin et al. (2006) demonstrated that pretreatment ^{18}F-FMISO was effective in determining hypoxic regions and tirapazamine was effective in patients with hypoxic tumours as assessed by ^{18}F-FMISO. Another group found that an standardised uptake value (SUV) > 2 and tumour to muscle ratio (TMR) > 1.6 at 4 h after injection were associated with disease recurrence after RT (Eschmann et al. 2005). The same group also investigated the changes in ^{18}F-FMISO uptake during RT by performing a scan pretreatment and repeating at 30 Gy. The mean SUV and TMR significantly decreased during RT, indicating RT-induced reoxygenation. Lee et al. (2009) also performed a baseline and midtreatment scan and found 90% of 20 patients had hypoxia before treatment and only 2 had detectable hypoxia on their midtreatment scan, which did not correlate with outcome. Zips et al. (2012) carried out a baseline scan and 3 further scans during RT. The imaging parameters from scans at week 1 and 2 of RT strongly correlated with progression free survival, suggesting the prognostic value of imaging hypoxia at the start of RT rather than at baseline. Smaller studies have showed variable results, with ^{18}F-FMISO showing borderline significance for stratifying patients into treatment outcome groups in 12 patients, and high SUV_{max} but not TMR correlating with poor DSS after RT or surgery in 17 patients (Kikuchi et al. 2011).

^{18}F-FMISO is not a universal hypoxia tracer in cancer. It has shown minimal activity in pancreatic cancer patients (Segard et al. 2013) and high background uptake in the normal rectum in patients with colorectal cancer, as well as tracer diffusion through the bowel wall, making images difficult to interpret (Roels et al. 2008). The low uptake of ^{18}F-FMISO in target tissue and slow clearance of unbound ^{18}F-FMISO from normoxic areas results in images of poor contrast. The relatively short half-life of 110 min hampers late imaging that could enhance contrast between hypoxic and normoxic tissues. This has led to the development of other tracers with improved pharmacokinetics.

3.6.5.3 ^{18}F-Fluoroazomycin Arabinofuranoside (^{18}F-FAZA)

This second generation 2-nitroimidazole is more hydrophilic than ^{18}F-FMISO and therefore has faster clearance from normal tissues and higher tumour to background ratios. Souvatzoglou et al. (2007) carried out an 11 patient pilot study in HNSCC and reported a higher contrast with non-target tissues compared to ^{18}F-FMISO, with an average TMR of 2.0 at 2 h post injection. Mortensen et al. (2012) performed static ^{18}F-FAZA imaging in 40 oropharynx SCC patients. A hypoxic volume could be identified in 25 patients, with a median TMR of 1.5. The distribution of hypoxia among HPV positive and negative tumours was not significantly different and there was a significant difference in DFS in patients with non-hypoxic tumours compared

with hypoxic tumours. Tumours were ranked according to the expression of genes included in a 15-gene hypoxia classifier but no correlation was found between the hypoxic status as assessed by the classifier and ^{18}F-FAZA imaging, despite the gene profile being validated using ^{18}F-FAZA autoradiography in human tumourxenografts (Toustrup et al. 2011).

3.6.5.4 Copper(II)-Diacetyl-Bis (N^4-Methylthiosemicarbazone) (Cu-ATSM)

Cu-ATSM was developed to overcome some of the limitations of ^{18}F-FMISO imaging. It is an uncharged lipophilic molecule of low molecular weight and high membrane permeability. It has fast tumour uptake and clearance form normoxic tissues, allowing rapid imaging after injection and higher hypoxic-to-normoxic contrast. It is also simpler to synthesise and radiolabel compared with ^{18}F-FMISO, but the longer half-life results in a higher radiation dose to patients (Lewis et al. 2001).

The hypoxia selectivity of Cu-ATSM is thought to be due to the bioreduction of Cu(II)-ATSM to Cu(I)-ATSM, which occurs in both normoxic and hypoxic cells, generating unstable anionic Cu(I)-ATSM. Cu(I) slowly dissociates from the ATSM ligand in cells with low oxygen concentration, becoming irreversibly trapped within the cell due to its negative charge. In the presence of oxygen Cu(I)-ATSM oxidised back to neutral Cu(II)-ATSM and diffuses out of the cell. The hypoxia selectivity therefore is a competition between dissociation and reoxidation of the reduced copper complexes, which is dependent on the oxygen concentration (Dearling et al. 2002). The precise cellular retention mechanism of Cu-ATSM is still unclear and uptake and retention has been associated with a number of other factors, such as over-reduced states caused by mitochondrial dysfunction (Yoshii et al. 2012), the expression of multidrug resistance protein (Liu et al. 2009) and Cu metabolism itself (Hueting et al. 2014). In addition preclinical studies have suggested hypoxia specificity may be dependent on tumour type (O'Donoghue et al. 2005; Yuan et al. 2006) and therefore concerns have been raised regarding the use of Cu-ATSM as a hypoxia radiotracer. However, small clinical studies in a number of tumour types have demonstrated correlation between uptake and poor prognosis, warranting further investigation.

3.6.5.5 Clinical Studies of Cu-ATSM in HNSCC

In 2001 Chao et al. (2001) used ^{60}Cu-ATSM in a radiotherapy planning study to demonstrate the feasibility of Cu-ATSM-guided IMRT to overcome hypoxia tumour resistance. Minagawa et al. (2011) assessed tumour hypoxia using ^{62}Cu-ATSM as a predictor of response in 17 patients. After 2 years of follow up SUV_{max} but not TMR was significantly different in patients with or without residual/recurrent tumour, and a $SUV_{max} > 5$ was a possible cut off threshold for

poor outcome. There was no significant difference in ^{18}F-FDG between patients with and without residual/recurrence tumour. Sato et al. (2014) also evaluated the prognostic potential of ^{62}Cu-ATSM in 25 patients with head and neck cancer. ^{62}Cu-ATSM $SUV_{max} > 3.6$ was associated significantly worse progression free survival (PFS), and a $TMR > 3.2$ was associated with significantly worse progression free survival and cause specific survival (CSS). ^{18}F-FDG uptake parameters did not distinguish patient with good and poor outcome. ^{64}Cu-ATSM in head and neck cancers was used to assess the efficacy of pretherapy ^{64}Cu-ATSM scanning as a prognostic factor of response to therapy in 11 patients (Grassi et al. 2014). Patients received a ^{64}Cu-ATSM and ^{18}F-FDG PET scan before commencing treatment and response was determined by an ^{18}F-FDG PET 3 months after therapy. The sensitivity and specificity of ^{64}Cu-ATSM SUV_{max} in predicting complete response to therapy were 100 and 50% respectively.

3.7 Conclusions

Tumour hypoxia remains one of the major causes of treatment failure in head and neck cancer. Hypoxia promotes a more aggressive tumour phenotype and is considered an important therapeutic target. Current methods of hypoxia identification are inefficient and therefore it is difficult to accurately assess hypoxia in tumours; accurate detection of hypoxia is important to allow stratification of patients for tailored treatment, and to reduce unnecessary side effects of intensive treatment modalities for less hypoxic tumours. In recent years, research in this field has substantially increased our understanding of the molecular pathways that contribute to tumour hypoxia and key pathways such as HIF, mTOR, UPR, and many more upstream and downstream biological targets of tumour hypoxia have been identified. Several of hypoxia-regulated genes are under investigation for drug development. In addition, a number of mRNA and microRNA signatures of tumour hypoxia have been published. So far, the clinical applications of such biomarkers have been limited due to inconsistencies and heterogeneity between studies. Approaches exploiting tumour hypoxia both for development of anti-cancer drugs that are activated in a hypoxic environment and therapeutic approaches that more directly target tumour hypoxia are under development.

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Chapter 4
Genetic Aberrations and Molecular Pathways in Head and Neck Cancer

Luis Monteiro and Saman Warnakulasuriya

Abstract In this chapter, we provide a comprehensive overview of common genetic aberrations so far discovered in head and neck cancer. We discuss how these genetic alterations may affect protein structure and function. We also highlight the latest discoveries in signalling pathways affected as a result of these genetic aberrations. A better understanding of these mechanisms underlying cancer formation, progression and resistance to therapy leads us to the next five chapters that discuss each of the key driver mutations and tumour suppressors that are lost in head and neck cancer.

4.1 Introduction

Carcinogenesis is based on non-lethal accumulation of successive genetic and epigenetic changes over many years, eventually transforming a normal cell (or a clone of cells) into a malignant cell. As discussed earlier in Chap. 1, in this multistep process certain groups of genes are particularly affected especially oncogenes and tumour suppressor genes, resulting in the acquisition of several important capabilities for tumour progression including independent growth and cell proliferation. Activation of oncogenes (e.g. EGFR, Ras, Cyclin D) may stimulate cell growth, or aberrations in tumour suppressors (e.g. p53, pRb, PTEN) may result in insensitivity to growth inhibition and avoidance of apoptosis. Several of these

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_4

molecular pathways lead to cell immortality, defects in DNA repair favouring genomic instability, neoangiogenesis where growth factors such as VEGF stimulate formation of new blood vessels permitting tumour progression, invasiveness and metastasis (Hahn and Weinberg 2002). Oncogenes are modified "normal" genes (termed proto-oncogenes) and are usually related with regulation of cell growth and differentiation. Several mechanisms can modify a proto-oncogene such as a mutation, gene amplification, or chromosome rearrangements leading to a growth advantage of the cell or its increased survival carrying such alterations. Tumour suppressor genes, by contrast, normally are involved in regulation and control of several pathways preventing deregulated cell growth. They could be altered by mutations, but also by deletion or epigenetic modifications. (Khan and Bisen 2013; Hahn and Weinberg 2002). Some of the genes known to be functionally altered in head and neck cancer (HNSCC) are discussed in this chapter.

4.2 EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein of 170 kDa comprising an extracellular ligand-binding domain, a hydrophobic transmembrane region and an intracellular tyrosine kinase domain. This receptor encoded by the *c-erbB* proto-oncogene located on chromosome 7 (7p12) is part of the *ErbB* family of receptor tyrosine kinase type I and contains four homologous receptors: ErbB1 (EGFR/HER-1), ErbB2 (HER-2), ErbB3 (HER-3) and ErbB4 (HER-4).

EGFR is a receptor expressed in various normal non-hematopoietic cells such as epithelial cells, the connective tissue cells, glial cells, smooth muscle cells and acini of the salivary glands. It is an important mediator receptor with actions such as growth, development, differentiation, and cell survival (O-charoenrat et al. 2002).

EGFR is a major driver of epithelial cell proliferation. Remarkably, EGFR is overexpressed in up to 90% of HNSCC, compared with levels in normal mucosa (Mao et al. 2004; Forastiere et al. 2001), and it is associated with aggressive disease and poor prognosis (Grandis et al. 1998; Chung et al. 2004). This receptor can be activated by various ligands such as epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amfiregulin (RA), epiregulin (ER), HB-EGF, and betacellulin. Ligand binding to EGFR induces the formation of receptor homo- or heterodimers with two members of the ErbB family and activation of the intrinsic tyrosine kinase activity at the internal side of the cell membrane, resulting in the phosphorylation of specific cytoplasmic tyrosine residues including the positions 992, 1068, 1086, 1148 or 1173, the most extensively and rapidly phosphorylated sites in EGFR (Monteiro et al. 2014).

These phosphorylated residues lead to activation of a series of pathways including mitogen-actived protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)/Akt pathway or phospholipase Cγ (PLCγ1) resulting in tumour-promoting cellular activities such as activation of cell cycle, enhancement of cell motility,

cytoskeletal changes, inhibition of apoptosis, and production of extracellular matrix-degrading enzymes (O-charoenrat et al. 2002).

Overexpression of EGFR has been described in several cancers, in particular carcinomas of the head and neck (80–100%), lung cancer (40–93%), breast cancer (14–91%), colorectal cancer (25–77%), kidney cancer (50–90%), bladder cancer (31–72%) in brain tumors including glioblastomas (40–63%), ovarian carcinomas (35–70%) or pancreatic cancer (30–50%) (Khalil et al. 2003).

EGFR protein is detected by immunohistochemistry in over 90% of HNSCC cases. We have previously shown both membranous and cytoplasmic expression of EGFR in oral squamous cell carcinoma (Monteiro et al. 2012). EGFR overexpression is mainly at the transcriptional level as there are only few EGFR—activating mutations in head and neck cancer. 10–30% of head and neck cancers display EGFR gene amplification, and a much lower proportion (1–7%) may have EGFR mutations. If found, the mutant form relates to EGFRvlll (Grandis and Tweardy 1993; Loeffer-Ragg et al. 2006; Temam et al. 2007; Sok et al. 2006).

The increased expression of EGFR referred to earlier is due to several mechanisms. The gene amplification, as with the *HER-2*, has been demonstrated in several neoplasms (Sauer et al. 2005). However, its frequency is low in cases with increased expression of EGFR suggesting that there could be other mechanisms involved (O-charoenrat et al. 2002). Activating gene mutations were found particularly in carcinomas of the lung, breast, head and neck and glioblastomas. Of these, point mutations were found in exon 21 (L858R), deletions in exon 19 (LREA) that result in the tyrosine kinase domain and deletions in exons 2–7, resulting in the *EGFRvIII* variant, lacking the extracellular binding domain but with constituent activation (Sok et al. 2006). In pancreatic carcinomas, prostate, lung and colon cancers the existence of an autocrine expression by a positive feedback mechanism with EGF and TGF-α, dramatically increase the EGFR expression (Yarden and Sliwkowski 2001). Increased expression of heterologous receptors such as HER-2 can provide greater stability and heterodimerization of EGFR.

EGFR is involved in tumour cell proliferation and cell growth as well as increasing cell life by its resistance to apoptosis. It may have effects on important stages of tumour invasion and spread, including promotion of cell motility, alteration and reduction of adhesion molecules such as integrins, E-cadherin, catenins β (described in detail in Chap. 1) and by stimulating proteolytic enzymes of extracellular matrix degradation such as metalloproteinases (e.g. MMP-9). The participation of the EGFR in angiogenesis, probably by regulating VEGF appears to be also relevant (O-charoenrat et al. 2002).

Activation of EGFR in malignant cells prolong its (cell) life. This attempt to cell immortalization is due in part to multiple kinases that determine the continuing proliferation but especially to the inhibition of apoptosis through pathways such as PI3K/Akt and STAT. The kinase Akt (protein kinase B) is also involved in cell proliferation and inhibits apoptosis inactivating the pro-apoptotic protein BAD and caspase-9. This pathway is negatively regulated by *PTEN* (phosphatase and tensin homolog deleted on chromosome TEN), an oncosuppressor gene that encodes a lipid phosphatase activity, located on chromosome 10 (10q23.3). Activated EGFR

can also recruit specifically for tyrosine residues dimers signal-transducing proteins and transcription activators referred to as STAT (Grandis et al. 2000). These dimers directly activate transcription of anti-apoptotic genes such as Bcl-xL and Mcl-1.

EGFR overexpression is shown in some studies to be associated with poor prognosis in particular colon cancer, breast cancer, lung and cancer of the head and neck (Grandis et al. 1998).

The determination of EGFR expression can be useful in planning the treatment with radiation or chemotherapy, since overexpression of this receptor is associated with increased radioresistance. Cancers that express EGFR have diminished DNA repair capacity and show evasion of apoptosis, so the choice of accelerated radiotherapy regimens may provide best results.

Increased EGFR expression in head and neck cancer makes this an attractive target molecule for therapeutic use of inhibitors of this receptor. Thus, considerable efforts have been made to target EGFR in HNSCC as discussed in Chap. 6. Numerous agents have been investigated including monoclonal antibodies, tyrosine kinase inhibitors, ligand-toxin conjugates or immunoconjugates (Sacco and Worden 2016). Completed and on-going anti-EGFR trials are presented in detail in Chaps. 6 and 10. Monoclonal antibodies act on the external domain of the EGFR blocking the growth factor binding and subsequent signal transduction. Cetuximab (IMC-C225), a chimeric antibody that interferes with ligand binding, was one of the first anti-EGFR antibodies to be studied. Cetuximab, was shown to prolong the median overall survival and reduced disease progression in patients with advanced HNSCC as part of combination therapies with radiation and chemotherapy (Bonner et al. 2006; Vermorken et al. 2008). These findings led to the FDA approval of cetuximab for using it together with radiation or as a single agent in patients that failed to respond to platinum-based therapy, and for recurrent or metastatic HNSCC in combination with standard chemotherapy (Vermorken et al. 2008). However, adding cetuximab to radiation and/or chemotherapy increases the overall response by ∼10–20% (Bonner et al. 2006; Vermorken et al. 2008), which is much lower than initially expected. Other antibodies that have been through clinical trials include panitumumab (ABX-EGF), zalutumumab (HuMax-EGFr), and others such as MDX-447 and ICR62 (Venook 2005). The tyrosine kinase inhibitors (TKIs) are small molecules which inhibit the tyrosine kinase activity of EGFR. Most TKIs cause a reversible inhibition, although there are some which cause irreversible inhibition and inhibition of multiple members of the ErbB receptor family receptors. These include gefitinib (ZD1839), erlotinib (OSI-774) and lapatinib (GW572016), thus targeting both EGFR and HER-2 (Venook 2005).

A further understanding of the EGFR signalling circuitry in HNSCC may reveal novel targets for HNSCC treatment, as a single agent or as part of new co-targeting options.

4.3 mTOR

PI3K/AKT/mTOR is one of the most frequently dysregulated signalling pathways in cancer, including oral cancer. This signalling pathway plays many roles in the cellular survival, migration, proliferation, and differentiation, as well as angiogenesis, protein synthesis, and glucose metabolism. PI3K/AKT/mTOR has been reported as an altered pathway in several cancers including oral cancers (Molinolo et al. 2009).

The mammalian target of rapamycin (mTOR) is a 290 kDa serine-threonine protein kinase, and an important downstream target of the PI3K/AKT signalling pathway involved in the regulation of overall cellular anabolism, cell growth, proliferation and survival. In response to multiple stimuli including nutrient, oxygen, insulin, growth factors, ATP, and tobacco carcinogens mTOR is activated by phosphorylation of Ser 2448, through the phosphatidylinositol 3-Kinase (PI3K)/AKT signalling pathway. This results in subsequent activation of three key modulators of protein biosynthesis: the eukaryotic translation factor 4E (eIF4E), the p70 ribosomal S6 kinase (p70S6 kinase), and elongation factor 2 (eEF2) (Xu et al. 2014).

Previous studies have shown that activated mTOR is deregulated and related to prognosis in several cancers including in oral cancer (Marques et al. 2016). Moreover, one of the most important aspects of mTOR is the possibility of using it as a target for anticancer therapy for tumours indicating its expression (see Chap. 7). Rapamycin analogs, including everolimus, temsirolimus, and ridaforolimus are small molecules that selectively inhibit mTOR activity. Several clinical trials for different cancers have revealed the clinical effectiveness of everolimus (Simpson et al. 2015). Additionally, recent interest has been reported on the anti-cancer effect of metformin, a classical anti-diabetic drug, by inhibition of mTOR activity. A multi-centre clinical trial is currently ongoing to demonstrate the benefits of metformin in reducing transformation of oral potentially malignant disorders (personal communication: Dr Silvio Gutkind).

4.4 RAS

The ras proteins correspond to a family of small GTPases that serve as cellular signal transducers inducing cell growth, migration, adhesion, cytoskeletal integrity, survival and differentiation. It was firstly described by Jennifer Harvey who observed that a group of genes from a murine leukemia virus (rat sarcoma virus) induced sarcomas in new-born rodents. Since then, the *ras* gene family is divided into three well-characterized genes: *Hras* (Harvey sarcoma virus-associated oncogene located on 11p15.5), *Kras* (Kirsten sarcoma virus located on 12p12.1) and *Nras* (Neuroblastoma derived located on 1p13.2) all encoding a ~21 kda molecular weight protein with guanosine triphosphate (GTP) activity (Murugan et al. 2012).

When a growth factor binds to tyrosine kinases receptor (TKRs), an active form of Grb2–SOS complexes and G proteins (guanine nucleotide-binding proteins)

induce ras activation. Then, *ras* stimulates several different effector pathways, most often the mitogen-activated protein kinase (MAPK). Active *ras* induces the phosphorylation by RAF1 kinase of both MEK1/2 in a specific threonine and tyrosine residues and subsequently the ERK1/2 resulting in cell growth and differentiation. *ras* also can activate the phosphatidylinositol-3-kinase (PI3K)/Akt pathway.

The *ras* gene is one of the most frequently mutated oncogene in cancer including oral cancer. Several types of mutations have been described in the *ras* family of protooncogenes (*K-ras*, *H-ras*, and *N-ras*), especially point mutations. In the case of mutations in codons 12, 13, or 61, the *ras* genes encode a protein that remains permanently activated which induces a continuous cell growth.

Mutations in the three isoforms of *ras* gene have been described in oral cancer but *Hras* mutation appears to be the most common when compared to the *Kras* and *Nras*. The reported prevalence of *Hras* mutations in oral cancer is 0–55% (the prevalence is reported to be high in tobacco chewers in south Asia—see later), *Nras* mutations 0–2.5%, and although most of the studies found no mutations in the *Kras* gene, the reported frequency is 0–33% (Murugan et al. 2012).

Ras could be overexpressed due to mutations but also by amplifications. *Hras, Kras* and *Nras* genes have been reported to be amplified in oral cancers. Interestingly, there are variations on reported prevalence of *ras* amplifications worldwide. *Hras* is amplified in western populations but few reports show gene amplification on Asian populations. This is true also for *ras* mutations confirming that there are possible geographic variation of the mechanisms of *ras* activation. Mutations of the *ras* genes were shown to be very uncommon in the oral cancers of western populations (Warnakulasuriya et al. 1992) while Asian populations who chew tobacco have a higher proportion of *Hras* mutations in codons 12 and 61, in their oral cancers (Saranath et al. 1991).

Although, mutational status of *ras* genes was determined long before EGFR was analysed their potential value in targeted therapy for oral cancer is presently limited.

4.5 p53

TP53 is a oncosuppressor gene located on the short arm of chromosome 17 (17p13.1) encoding a phosphoprotein of 53 kD—p53, with main functions: the regulation of gene transcription, regulation of DNA synthesis and repair and apoptosis (Lane 1992).

This protein consists of 393 amino acids and divided into five areas, each with a different structure and function. The first area is the N′ terminal portion called the transcriptional activation domain, is responsible for recruiting the necessary machinery for new mRNA synthesis. On the other hand, it also contains the binding site of MDM2 protein. The second domain rich in proline is important for cell cycle arrest function of p53 and apoptosis. It has also the codon 72 polymorphism region that gives rise to two functionally distinct variants of the protein known as p53

(Pro) P and p53 (Arg) R. The central domain of the protein, the DNA binding domain is responsible for specific binding to certain gene promoters, such as the p21 or Bax. Much of the p53 mutations affect this area preventing this interaction. The oligomerization domain allows interactions and formation of the p53 tetramer. The last area is the C' terminal region that is responsible for interaction with the DNA-binding domain thereby negatively regulating p53's ability to bind certain genes maintaining p53 in an inactive form. Phosphorylation of this zone allows the free DNA binding region thereby activating p53. It is also a zone of recognition of DNA errors (Partridge et al. 2007).

p53 is a transcription factor present in most cells capable of detecting cellular stress signals including errors in DNA due to radiation damage, oncogene activation, hypoxia, oxidative damage, inducing a set of adaptive responses of cell protection by activating certain genes. It is essential to avoid inappropriate cellular proliferation, blocking proliferation at different phases of the cell cycle, and in the participation of correction of genetic errors or inducing apoptosis.

In normal cells p53 level is very low, reflecting the short half-life of the protein (5–20 min). However, in the presence of cellular stress its level increases dramatically. Monomers are associated in the cytoplasm forming tetramers which bind to various transcription elements in the core there by activating certain genes, initiating a cell protective response. Hence this protein is designated as a guardian of the genome (Lane 1992).

Several genes activated by p53 are responsible for cell cycle arrest in G1 and G2 checkpoints and during mitosis. An increase in p53 CDKN1A stimulates transcription of the gene encoding the p21 protein, responsible for cell cycle arrest at the G1 phase. This protein complex inhibits CDK4/6—cyclin D1 stopping cell cycle progression in the transition phase G1/S. Thus, E2F and pRB proteins prevent transcription factors required for cell cycle and cyclin E and PCNA. p21 also directly inhibits cyclin E/CDK2 complex and PCNA by blocking the DNA polymerase activity. This regulation can also occur in the G2/M transition with the complex inhibition CDK1/cyclin B1. Besides p21, this stage can also activate p53 directly by 14-3-3σ protein that binds to the complex CDK1/cyclin B1-exporting the core and *GADD45* gene complex dissociates preventing mitosis (Partridge et al. 2007). The pause in the cell cycle allows DNA repair that can be stimulated by transcription of genes *GADD45* in association with PCNA, p48/DDB2, ERCC2 and ERC3. In cases of severe injury or DNA repair inability p53 induces apoptosis through the activation of genes such as Bax, PIG3, Puma, Noxa, Fas, TRAIL, PTEN and suppressing anti-apoptotic genes such as *Bcl-2, BclxL or survivin*. If the DNA repair is successful, the active MDM2 promotes p53 degradation releasing the cell cycle (Partridge et al. 2007).

Many aberrations of p53 occur in several types of tumours and reported to be close to 50% of the cases by mutations of its gene. These mutations can be missense where a wrong nucleotide is inserted into the DNA chain by changing a codon for a given amino acid with the production of a complete but non-functional protein. Other mutations include frame shift mutations leading to a stop codon causing a truncated protein. Most of the mutations occurs between exons 4 and 9 notably at

codons 238–248 (exon 7), and 278–281 (exon 8). These mutations are located in hotspot areas in the DNA binding domain preventing binding to p53 target genes. Most of the mutations in the head and neck carcinomas occur in guanine nucleotide (G) and are associated with carcinogens present in tobacco.

However, other mechanisms also may lead to loss of p53 function even in the presence of a normal protein. This may occur in tumours with increased expression of MDM2 caused by amplification or polymorphism of this gene. This protein binds to the p53 N-terminal region and blocks its ability to function as transcription factor. Then, it carries out the core leading to its degradation by the ubiquitin proteasome system. The control of the MDM2 levels is carried out by a p14 protein that binds to MDM2, leading to degradation thereby releasing p53.

The p53 function can be compromised by infection with the human papillomavirus type 16 (HPV 16). The viral E6 protein competes with MDM2 to sequester p53 transporting the ubiquitin proteosome complex for degradation.

The *TP53* gene mutations with consequent and frequent p53 overexpression represent one of the most reported molecular changes in squamous cell carcinomas of the oral cavity observed in more than 50% of cases (Oliveira et al. 2008). In a review of studies reporting on p53 overexpression in potentially malignant disorders an increase in p53 expression was observed in 47% of cases (Warnakulasuriya 2000). Suprabasal expression of p53 increases the risk of malignant transformation.

The usefulness of studying the p53 expression analysis of surgical margins from patients with oral carcinoma indicating residual disease as well as the likelyhood of field cancerization is reported by several authors (Bilde et al. 2009).

Increased p53 expression was shown to be associated with aggressive behaviour in the presence of metastases and poorer survival in patients with squamous cell carcinomas of the oral cavity (Oliveira et al. 2008). The existence of Arg72 polymorphism was associated with increased apoptotic index, with a good response to radiotherapy and chemotherapy. The same polymorphism may indicate the susceptibility to oropharyngeal infection by HPV 16. The role of p53 in targeted therapies is discussed further in Chap. 5.

4.6 pRb

pRb is one of the most important tumour suppressor proteins that controls whether cells progress to cellular division or not. pRb protein is encoded by the *RB1* gene located on 13q14.1-q14.2. This gene was firstly discovered in retinoblastoma cancers where the inactivation of both alleles were linked to retinoblastoma occurrence. Subsequently, pRb inactivation was observed also in other cancers such as breast, lung, prostate, and bladder cancer. Rb inactivation in oral cancer has not yet been reported, but it is possible to be joined with *TP53* inactivation in some cases of oral cancer (Khan and Bisen 2013).

In the normal cell, pRb is in a hypo-phosphorylated state, linked to E2F transcription factors that no longer can activate DNA synthesis and subsequently cell

cycle progression. When a mitogenic stimulus is produced and transmitted by oncoproteins such as c-Myc and Ras protein, there is transcription of cyclin A, D, E which then bind and activate CDKs. As a result of this activation CDKs level rises dramatically leading to the phosphorylation of pRb. Now, phosphorylated pRb releases the E2F factor permitting the transcription of genes involved in DNA replication and cell cycle progression (Khan and Bisen 2013).

Although, pRb aberration is rare in oral cancers, most of them lose the pRb function by activation of other proteins such as p16, an inhibitors of cyclin-bound cyclin-dependent kinases (CDKs) CDK4 and CDK6.

4.7 VEGF

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is an important step for the growth and metastasis of solid tumours. This concept is based on the fact that solid tumours cannot exceed 1–2 mm^3 without an adequate blood supply. A variety of molecules involved in tumour angiogenesis have been identified. Among them, vascular endothelial growth factor (VEGF) plays a major key role including induction, proliferation, migration and survival of endothelial cell and capillary tube formation and is correlated with increased neovascularisation within tumoral cells.

VEGF is a binding glycoprotein heparin biologically produced in active form by tumour cells, endothelial cells and macrophages by stimulation of TGF-α, TGF-β, PDGF, HIF-1B (under hypoxic conditions). Its functions are: inducing increased vascular permeability, regulates the production of proteases, migration, proliferation and differentiation and enhances endothelial cell survival by inhibiting apoptosis (Neuchrist et al. 2001; Vassilakopoulou et al. 2015).

The importance of VEGF is demonstrated in cases with mutations or absence of its the gene resulting in the lack of proper vascularization.

VEGF expression is increased in the majority of cancers studied so far, including haematological malignancies, colon and rectal cancers, hepatocellular carcinoma, lung, thyroid, breast, kidney, bladder, ovarian and uttering cervix carcinomas, and head and neck and intracranial tumours. VEGF expression in the primary tumour has been correlated in many studies with a greater risk of recurrence and poor prognosis. The prognostic influence of the presence of VEGF was observed in various tumours including colon, breast, stomach, lung and head and neck cancers. The association with nodal metastases has also been described (Zang et al. 2013).

The action of VEGF is mediated by three types of tyrosine kinase receptors: VEGFR-1 or FLT-1 (fms-like tyrosine kinase), VEGFR-2 or FLK-1/KDR (fetal liver kinase-1/kinase insert domain receptor) and VEGFR-3 or FLT-4.

4.7.1 Vascular endothelial growth factor receptors (VEGFR)

The family of VEGFR is represented by 3 receptors with tyrosine kinase activity known as VEGFR-1 (Flt-1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt-4). These receptors have an extracellular domain consisting of immunoglobulín sequences, a transmembrane domain and intracellular domain with tyrosine kinase activity. VEGFR-1 and VEGFR-2 are located on the vascular endothelial cells, circulating monocytes and macrophages. Both were detected in melanoma tumour cells, malignancies of the pancreas, ovary, and head and neck. VEGFR-3 is mostly found in vascular endothelia of lymphatic vessels but also have been found in head and neck tumour cells and in Kaposi's sarcoma. Expression of VEGFR-1 and VEGFR-3 was also observed in stromal fibroblasts in tumours.

VEGFR-2 works as a recruiter of hematopoietic and endothelial precursor cells from bone marrow (Neuchrist et al. 2001). VEGFR-3 is mostly involved in lymphangiogenesis and this may probably relate to its metastatic effect. Hiratsuka et al. (2002), have shown that VEGFR-1 expression in monocytes and macrophages may have a peculiar effect on tumour dissemination. Using animals with intact VEGFR-1 tyrosine kinase activity and other animals without such activity it was found that the former had more MMP-9 and increased presence of monocytes and macrophages in lung tissue before the onset of pulmonary metastases which led the authors to theorize that infiltration of macrophages occur via VEGFR-1 in the lung. An increase in MMP-9 expression creates a pre-metastatic niche predisposing to lung metastasis (Hiratsuka et al. 2002).

The role of angiogenesis in tumour progression and spread, controlled by existing cellular factors and receptors in the tumour microenvironment makes these molecules attractive therapeutic targets.

Inhibitors (antibodies and selective inhibitors) of VEGF and VEGFR are already available and are undergoing clinical trials (for details see Chap. 10). Bevacizumab was one of the first VEGF antibodies to be used and approved for anti-angiogenic therapy in combination with first line chemotherapy for several types of cancers, including cancer of the lung. Anti-angiogenic therapy can also improve tumour response to radiation in head and neck cancers. Several tyrosine kinase inhibitors of multiple receptors are in various clinical trial phases and include vandetanib, sorafenib or sunitinib (Hsu et al. 2014). VEGFR-1 ribozymes, VEGF toxin conjugates and soluble VEGF receptors are also being investigated.

4.8 Survivin

The avoidance of apoptosis by tumour cells is one of the hallmarks of malignancy. Several proteins are involved in regulation of apoptosis such as the "inhibitor of apoptosis proteins" (IAPs), including XIAP, cIAP-1/-2 and survivin (Lippert et al. 2007).

Survivin protein, derived from chromosome 17q25, is a subunit of the chromosomal passenger complex (CPC), composed of the mitotic kinase Aurora-B, Borealin and INCENP. This complex participates in the mitotic checkpoint preventing errors between chromosomes and the mitotic spindle. Survivin is undetected in normal mucosa but is overexpressed in oral cancers leading to chemo- and radiotherapy resistance and reduced patient survival (Lippert et al. 2007). Gene silencing is one of the approaches tried so far in survivin-based therapy (see Chap. 8).

4.9 NOTCH Pathway

Notch signaling is an evolutionarily conserved pathway that influences cell proliferation, apoptosis and differentiation of diverse types of cells in a variety of organisms. In mammals there are four receptors (Notch1–4) and five ligands (JAGGED1, 2 and DLL1, 3 and 4) (Kayamori et al. 2016). Activation of Notch signaling requires binding of its ligands, Jagged and Delta-like (DLL) followed by proteolytic release of the Notch intracellular domain (NIC) and its translocation to the nucleus, which initiates transcription of the NOTCH target genes (Hijioka et al. 2010).

Recent studies have shown that dysregulation of Notch pathway is intricate in diverse diseases, including various types of cancers, as in T-cell acute lymphoblastic leukemia, non-small cell lung cancer, ovarian carcinomas, colon cancer, pancreatic cancer, osteosarcoma, and head and neck squamous cell carcinoma (HNSCC) (Yap et al. 2015; Song et al. 2014).

Recently, exome sequencing has revealed *Notch-1* as the second most frequently mutated gene in HNSCC after *TP53* (Agrawal et al. 2011). *Notch-1* mutation comprises about 50% of activated mutations in the Chinese population and 10% in the Caucasian population. Song et al. (2014) suggested that use of high alcohol-containing beverages in China could contribute to some of these divergent findings. *Notch-1* mutations were also found in Chinese patients with oral leukoplakia and invasive oral cancers (Izumchenko et al. 2015).

Yoshida et al. (2013) evaluated Notch-1 immunohistochemistry expression in 54 OSCC cases and verified that Notch-1 expression was positively correlated with T-stage and clinical stage, suggesting that increased expression of Notch-1 may correlate with OSCC progression. In another study by Hijioka et al. (2010) the expression of Notch pathway molecules in both OSCC cell lines and biopsy samples were analyzed, showing that Notch1, Notch2, Jagged1 and the Notch

targets HES1 and HEY1 were upregulated. In addition, γ-secretase inhibitor (GSI), a pharmacological agent known to effectively block Notch activation, prevented the in vitro growth of OSCC cells. With the same inhibitor, Zhang et al. (2011) demonstrated in HNSCC xenografts in nude mice a reduction of cancer stem-cells, self-renewal and consequently a delay in tumorigenesis.

Though the oncogenic role of Notch signaling is still in debate, recent data from exome sequencing studies conducted by Agrawal et al. (2011) revealed a tumour suppressor role for NOTCH in HNSCC by identifying loss-of-function mutations in the *NOTCH1* gene in a significant proportion of patients-in nearly 40% of the 28 mutations identified. In a recent review on sequencing the head and neck cancer genome by Sun and Califano (2014) the authors placed emphasis on the therapeutic implications of genes frequently altered in HNSCCs (i.e., TP53, PIK3CA, and NOTCH1) and their corresponding pathways, with a particular focus on recent findings of Notch signaling pathway activation in HNSCC.

4.10 Conclusions

Most cancers are sporadic in that they are caused by genetic changes that happen mostly by chance and are not inherited. They are acquired sometime during a person's lifetime. In most cases, a mutation within a gene does not lead to the development of cancer. Although a large number of somatic alterations are observed, only a fraction of these alterations drive cells to uncontrolled growth and development of resistance to treatment. Development of cancer requires multiple mutations to occur within several key genes, including mutations in proto-oncogenes, tumor suppressor genes, and DNA repair genes. In this chapter we have presented the common genes that are mostly altered in head and neck cancers. The paucity of driver mutations and more frequent losses of tumour suppressor genes do pose a challenge for development of targeted therapy. As presented in detail in later chapters few of these aberrations are potential targets for molecular based therapies that are currently in development.

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Chapter 5
p53 in Head and Neck Squamous Cell Carcinoma

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Abstract Head and neck squamous cell carcinoma (HNSCC) is the 6th most common cancer worldwide and has a mortality rate of 50% despite surgery. The major risk factors associated with HNSCC are smoking, alcohol, and human papillomavirus (HPV). p53, a transcriptional factor, is the most commonly mutated protein in HNSCC and plays an important and early role in tumorigenesis. The chapter highlights the story of p53 in the progression and management of HNSCC. In particular, we address p53's mutational landscape and its resultant phenotypic outcomes. In addition, p53 as a prognostic biomarker and predictive biomarker for clinical outcome is addressed. Finally, we discuss p53 as a druggable target in HNSCC patients. This chapter aims to expand the understanding of the role of p53 in HNSCC in order to improve management of HNSCC patients by providing them with a personalized and customizable treatment plan.

5.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) has an incidence of about 600,000 cases making it the 6th most common cancer worldwide (Ferlay et al. 2010). Major risk factors associated with HNSCC include smoking, alcohol, and infection with viruses such as Human papilloma virus (HPV) (Argiris et al. 2008). These risk factors are closely tied with the molecular tumorigenesis of HNSCC. HNSCC is thought to be a result of a series of genetic changes. One of the most important genes implicated in the development of HNSCC includes the TP53 gene (Agrawal et al. 2011). Although HPV-positive and HPV-negative HNSCC have

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_5

distinct biological features as evidenced by genome wide sequencing and epidemiological differences, the TP53 gene plays an important role in the tumorigenesis of both cancers (The Cancer Genome Atlas 2015; Ang et al. 2010). Prognostic stratification of HNSCC has been done by anatomic site, stage, grade, and HPV status, yet the mortality rate in HNSCC remains at 50% despite surgery, chemotherapy, and radiation therapy in these patients (Ferlay et al. 2010). There exists a need to further explore biomarkers in order to better stratify HNSCC by prognosis and to provide a personalized treatment plan for every HNSCC patient. In particular, the utility of p53 as a prognostic marker and the value of p53 as a druggable target by genetic and chemical approaches have been areas of interest in HNSCC.

In this chapter, we highlight the story of p53 in the molecular pathogenesis of HNSCC both in HPV-positive HNSCC and in HPV-negative HNSCC. We explore the mutational landscape of TP53 and its subsequent effect on tumor phenotype. The mutational landscape also highlights the utility of TP53 as a predictive biomarker for clinical outcome. Finally, we review the therapeutic approaches and implications of p53 in HNSCC.

5.2 Normal Function of p53

The TP53 gene codes for p53, which is a tumour suppressing transcriptional factor that regulates cell cycle progression and apoptosis in response to environmental stressors (Vousden and Lane 2007). It is helpful to imagine p53 as the centre of a complex set of interactions between upstream regulators, downstream regulators, and stressors. The upstream regulators increase level of p53 transcriptional activity, leading to regulation of downstream signalling, inducing an appropriate response via target gene transcription (Brown et al. 2009). In normal resting conditions, murine double minute 2 (MDM2), which is an E3 ubiquitin ligase, targets p53 for ubiquitin-dependent degradation and inhibits its activity (Bond et al. 2005). MDM4, an MDM2 analogue, also binds to MDM2 creating a heterocomplex potentiating the effects of MDM2 (Linares et al. 2003). In contrast, ARF inhibits the function of this complex, leading to stabilization of p53 and enhancing its function (Zhang and Xiong 2001). In stressed conditions, the MDM2-MDM4 heterocomplex can undergo phosphorylation or degradation leading to activation of p53 (Wang et al. 2009). This concept is illustrated in the p53 response to ionizing radiation, which leads to MDM2-MDM4 heterocomplex degradation and p53 activation (Wang et al. 2009). Other stressors such as DNA breaks, hypoxia, and UV irradiation induce a similar molecular response resulting in activation of the p53 program (Vousden and Lu 2002). The activated p53 leads to complex downstream effects via gene transcription, including growth arrest (p21, GADD45, PRPM), apoptosis (Scotin, KILLER, FAS, BBC3, TP5313), prevention of angiogenesis (THBS1), and DNA repair (ST13) (Brown et al. 2009).

In addition to these transcriptional downstream effects of p53, there is a myriad of evidence proposing additional non-transcriptional functions of p53 in regulating cell function. These additional non-transcriptional functions include maturation of growth-suppressive miRNAs, mitochondrial-associated role in apoptosis, and inhibition of cellular metabolism pathways such as aerobic glycolysis, lipid synthesis, and the pentose phosphate pathway (Berkers et al. 2013; Brown et al. 2009; Yu and Zhang 2003; Suzuki et al. 2009). Interestingly, the non-transcriptional functions of p53 may be sufficient for the tumour-suppressive effects of p53 and do not require the downstream transcription regulators highlighted above (Zhou et al. 2016).

5.3 Mutations of TP53 in HNSCC and Subsequent Effects

TP53 resides on chromosome 17p13.1 and is the most commonly mutated gene in head and neck cancer. TP53 is mutated in approximately 50–60% of head and neck squamous cell carcinomas (HNSCC) (Agrawal et al. 2011; Braakhuis et al. 2004). Specifically, this percentage is increased to up to 78% in HPV-negative tumors (Agrawal et al. 2011). Missense mutations are the most common mutations in the TP53 gene leading to a single amino acid change and change of function of TP53 gene (Agrawal et al. 2011). The most common missense mutation occurs in arginine residues in the core domain. Glycine and cysteine are the second most common mutated residues (Bullock et al. 2000). Specifically, molecular epidemiology data has described codons R175, H179, R196, R213, G245, R248, R273and R282 as the most common locations of missense mutations in TP53 (Fig. 5.1) (Balz et al. 2003; The Cancer Genome Atlas 2015). Compared to other cancers, the frequency of TP53 mutations is higher in HNSCC (Hussain and Harris 1998), which implies that TP53 mutations are selectively advantageous in the carcinogenesis of HNSCC (Sigal and Rotter 2000). In addition, approximately 95% of these mutations occur in

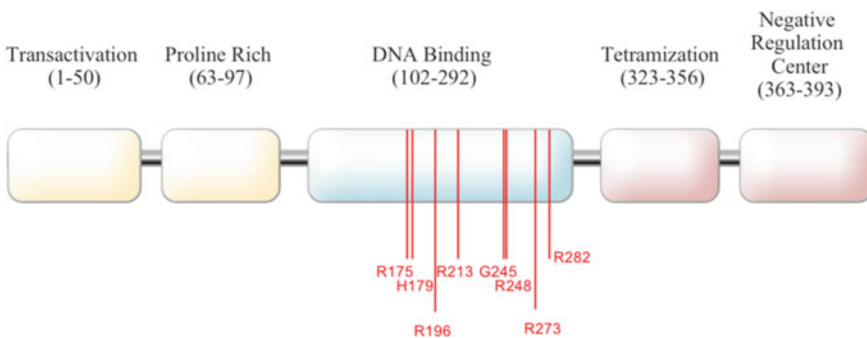

Fig. 5.1 The most prevalent hotspot mutations in p53 in head and neck squamous cell carcinoma occur in the DNA binding domain (TCGA n = 510)

the core DNA-binding domain of this gene, highlighting the effect on the function of TP53 as a transcription factor in HNSCC (Brown et al. 2009; Hainaut and Hollstein 2000).

Mutations in TP53 are divided into two categories: structural and DNA contact (Bullock et al. 2000). Structural mutations, such as V143A, change the conformation of the p53 protein and can lead to global denaturation of protein, preventing it from binding to the DNA appropriately (Bullock et al. 2000). DNA contact mutations, such as R273H, change the amino acid in the DNA-binding domain, altering the ability of the p53 protein to bind to the appropriate p53 response element in the DNA without altering its structure (Bullock et al. 2000). Moreover, DNA contact mutations can also lead to structural changes. Mutation of one TP53 allele is usually sufficient to compromise p53 function by exerting a dominant-negative effect on the wild-type allele (Milner and Medcalf 1991). This is due to the co-translational effect of mutant p53 on wild-type conformation, which depends on the interaction between mutant nascent polypeptides and oligomerization of the wild-type p53 full length proteins (Milner and Medcalf 1991). This induces the wild-type p53 protein to adopt a mutant conformation in a cooperative manner. The dominant-negative effect therefore suppresses the wild-type p53 protein from exerting its tumor suppressive functions (Milner and Medcalf 1991). The dominant-negative effect of mutant p53 on wild-type p53 has been described in various cellular processes such as apoptosis, growth arrest, and development (Aloni-Grinstein et al. 1995; Gottlieb et al. 1994).

TP53 mutations are early contributors to the molecular carcinogenesis of HNSCC. Leemans et al. describe at least three genetic subclasses of HNSCC: HPV-associated HNSCC, HPV-negative HNSCC with high chromosomal instability (high CIN), and HPV-negative HNSCC with low chromosomal instability (low CIN) (Leemans et al. 2011). These have been described based on the difference in molecular pathogenesis. Leemans et al. also described a hypothetical model of the molecular carcinogenesis of HNSCC stratified based on the genetic subtypes highlighted above. Their model was based on three steps of carcinogenesis, including the conversion of an adult stem cell to a mutated clone expressing uncontrollable growth, the transformation event which turns these clones into an overt carcinoma, and the development of metastasis (Leemans et al. 2011). Specifically, early in the pathogenesis a stem cell acquires one or more genetic mutations, including a mutation in TP53, which reproduces genetically altered daughter cells that have acquired said mutant TP53 (Leemans et al. 2011). These mutations allow for a selective advantage expanding a clonal unit into a field and replacing normal tissue (see Chap. 2). Subsequently, this subclone can invade basement membrane and lead to metastasis. As highlighted, TP53 mutations occur early, and are therefore essential, to the progression to overt HNSCC by contributing to genomic instability and clonal expansion (Braakhuis et al. 2003; Zhou et al. 2016).

It is helpful to understand the functional phenotypical consequence of early TP53 genetic alterations in the context of HNSCC. A conditionally immortalized model of oral keratinocytes in vitro was used to assess functional implication of

TP53 mutations in pathogenesis of HNSCC (Smeets et al. 2011). Inactivation of p53, either by knock down of TP53 with short hairpin RNA (shRNA) or by expression of dominant-negative mutant p53 in R172H codon, extended cell life span (Smeets et al. 2011). The cells became immortal with the combination of $p16^{INK4A}$ knockdown, ectopic cyclin D1, or HPV16 E7(Smeets et al. 2011). This cancer-associated phenotype of cellular immortalization aligns with the timing of the early genetic alteration in TP53 in the pathogenesis of HPV-negative HNSCC (Leemans et al. 2011). It is important to note that not all HNSCCs have TP53 mutations or have HPV associated degradation of p53. Therefore, a subset of HNSCC can be explained by the existence of a p53-independent pathway, or a p53-dependent pathway independent of p53 mutation (Berns et al. 2004; Leemans et al. 2011).

The above description of the nature of mutations in TP53 has led to the development of the gain of function (GOF) hypothesis. The GOF hypothesis highlights that a mutation in the gene does not just lose its wild-type function, but instead this mutation allows for positive selection due to its advantageous nature in the process of carcinogenesis (Freed-Pastor and Prives 2012). Tumour suppressor genes usually gain deletion or non-sense mutations that lead to deleted or truncated proteins (Levine et al. 1995). In contrast, mutations in TP53 are missense mutations that lead to either a change in structural conformation or DNA binding ability of a full-length protein. In addition, TP53 mutations in one allele exert a dominant-negative effect on the wild-type allele, highlighting the advantageous state of losing heterozygosity in favor of tumorigenesis (Brosh and Rotter 2009).

In addition to the above observations of missense mutations and dominant-negative effect on the wild-type alleles, there exists evidence that mutated p53 protein can exert oncogenic effects independently of its effects on wild-type p53 (Sigal and Rotter 2000). Some of the most important evidence to support of the GOF hypothesis has been done in mice models. In comparison to heterozygous or null mice, animals with one mutant TP53 showed a wider tumour spectrum with distant metastasis (Olive et al. 2004; Lang et al. 2004). These mutant-p53-driven cancers highlighted a mouse model of Li Fraumeni Syndrome in an attempt to understand the human disease (Olive et al. 2004; Lang et al. 2004). Oncogenic functions of mutant p53 include invasion, migration, scattering, angiogenesis, stem cell expansion, survival, proliferation and tissue remodelling (Muller and Vousden 2013).

It is important to highlight the mechanisms by which mutations harbour GOF activities. Multiple authors have categorized the mechanisms leading to GOF. The three essential mechanisms include (1) interaction between mutant p53 and other proteins leading to inhibition or enhancement of the function of target gene, (2) interaction between mutant p53 and other proteins not related to gene transcription, leading to enhancement or inhibition of their function, and (3) regulation of transcription via binding to novel genes (Muller and Vousden 2013).

The first model highlights p53's participation in complex interactions with different proteins to enhance gene transcription. Some of the proteins implicated in these interactions include NF-Y, Sp1, Ets-1, VDR, SREBP-2, and PML (Freed-Pastor and Prives 2012). The interaction between p53 and these proteins

depends on the mutated codon in p53 and resulting altered protein. For example, p53 with mutations in codons R175H or R273C bind to transcriptional factor nuclear factor Y (NF-Y), along with transcriptional cofactor p300 to deregulate cell cycle checkpoints after low levels of DNA damage (Freed-Pastor and Prives 2012). DNA topoisomerase 2-binding protein 1 (TopBP1) also plays a role in this complex interaction to induce gene transcription (Freed-Pastor and Prives 2012; Liu et al. 2011).

The first model also contains mutant p53 complex interactions with proteins to inhibit their gene transcription. Some of the most studied proteins with which p53 interacts are p63 and p73. p53, p63, and p73 are part of the same family due to the high degree of similarity in their amino acid sequences (Li and Prives 2007; Freed-Pastor and Prives 2012). All three proteins are expressed in multiple isoforms complicating their interactions. p63 and p73 can combine forming homo or heterotetramers, but are unable to bind with wild-type p53 (Davison et al. 1999). TP53 mutated at codons R1775H, Y220C, R248 W are responsible for a particular mutant p53 that allows for interaction with p63 and p73. Interactions between these p53 family members are regulated by other proteins including TopBP1 (Liu et al. 2011). This model of mutant p53 interactions with p63 and p73 has been implicated in many GOF activities including invasion and metastasis of tumor (Muller et al. 2009; Adorno et al. 2009). Specifically, in HNSCC, Valenti et al. found that mutant p53 recruits E2F creating a complex that binds to the promotors of BRCA1 and RAD17 and prevents their transcription (Valenti et al. 2015). This causes accumulation of DNA damage and genomic instability leading to tumorigenesis (Valenti et al. 2015).

In the second model, mutant p53 binds with proteins to change their function without directly affecting gene transcription. An example of this is the interaction between mutant p53 (R248W and R273H) and MRE11, a DNA nuclease involved in DNA repair (Song et al. 2007). This interaction blocks both the recruitment of the MRE11-Rad50-NBS complex to DNA double-stranded breaks and phosphorylation of ataxia telangiectasia-mutated (ATM), there by promoting genetic instability (Song et al. 2007).

In the last model, mutant p53 can bind to novel genes and induce transcription. Mutations in DNA binding domain of TP53 alter the binding ability of p53 to a p53-response-element and instead enables binding to a currently unidentified mutant p53-response-element (Strano et al. 2007). Mutated p53 proteins are involved in transcription of many genes leading to functions such as increased proliferation, chemoresistance, disruption of metabolism, and mRNA processing (Freed-Pastor and Prives 2012; Di Agostino et al. 2008; Sankala et al. 2011; Freed-Pastor et al. 2012; Girardini et al. 2011).

The GOF hypothesis illustrates multiple critical points. The complexity of interactions requiring multiple proteins, transcription factors, cofactors, and external stimuli highlights the significance of cellular context in the exact GOF activity. In addition, certain different GOF activities are dependent on specific mutations in specific codons. Finally, it is important to note that the three models are not completely distinct, but instead can overlap significantly between one another.

5.4 TP53 as a Prognostic Marker

From the previous information highlighted above, it seems intuitive that TP53 can be a prognostic indicator in HNSCC since TP53 mutations are involved in most HNSCCs. In addition, the myriad of TP53 mutants and their varied resulting proteins have shown to lead to a varied change in function of the p53 protein. Due to these observations, it seems plausible that p53 mutational status will have utility as a prognostic biomarker.

Immunohistochemistry has yielded controversial results in assessing p53 as a biomarker in HNSCC (Szentkúti et al. 2015; Bradford et al. 2003). Some authors have found no correlation between p53 expression and prognosis, while others have found a negative correlation (Szentkúti et al. 2015; Bradford et al. 2003). Poeta et al. (2007) described failure of this technique due to its inability to detect frame-shift, splice-site, and null mutations as well as these specific mutations' clinical phenotype. A recent review identified the inconsistent use of antibodies to detect p53 in immunohistochemistry (Tandon et al. 2010). The investigators also attributed an inconsistent definition of p53 positivity versus p53 negativity in these studies (Tandon et al. 2010). Lastly, immunohistochemistry fails to differentiate between overexpression of p53 due to a mutation or due to genotoxic stress (Tandon et al. 2010). Thus, immunohistochemistry is limited in its ability to detect p53 mutations and therefore cannot be used to correlate p53 mutations with clinical outcome.

New evidence has suggested that immunohistochemical analysis of p53 as a prognostic marker is valuable when added to other biomarkers. Recently, SET and MYND domain-containing protein 2 (SMYD2) , an oncogene, was shown to inhibit p53 through the methylation of lysine 370 (K370) leading to cell cycle dysregulation (Huang et al. 2006). The combination of p53 and SMYD2 identified via immunohistochemical analysis was found to be a significant prognostic indicator in HNSCC (Ohtomo-Oda et al. 2016). Another study analyzing six surrogate markers (epidermal growth factor receptor (EGFR), p53, Bcl2, CD117, keratin 5 and E-cadherin) found $EGFR^{+}$/p53+/$bcl2^{-}$ sub-class showed significant correlations with grade and TNM parameters (Cimpean et al. 2016). While trying to understand the reason behind the prognostic implication of this combination, the authors concluded that mutant p53 can down regulate bcl2 (Cimpean et al. 2016). Mutant p53's interaction with EGFR is less understood, but the authors have indicated that positive p53 enhances sensitivity of tumour to EGFR inhibitors indicating a significant interaction between p53 and EGFR. Therefore, although there might be inherent limitations to immunohistochemistry in detecting p53 mutations and subsequent clinical relevance, there is still some prognostic value in detecting p53 by immunohistochemistry in combination with other biomarkers.

Rapid mutation analysis, in contrast, can detect mutational changes more accurately and specifically allowing a more comprehensive understanding of TP53 mutations' relevance in clinical outcome. Poeta et al. (2007), studied the incidence of TP53 mutations in HNSCC and associations between TP53 status and survival

using rapid mutational analysis. They conducted the analysis by looking at the entire coding region of TP53, from exons 2 to 11. The investigators classified different mutations in two categories named ‘disruptive’ and ‘non-disruptive’ mutations (Poeta et al. 2007). Disruptive mutations are nonconservative mutations located inside the key DNA-binding domain (L2–L3 region), or stop codons in any region, and nondisruptive mutations are conservative mutations or nonconservative mutations outside the L2–L3 region (excluding stop codons). They found that any mutant TP53 associated HNSCC indicated a worse prognosis than wild-type p53 associated HNSCC. In particular, they found that disruptive mutations indicated an even worse statistically significant prognosis, even after adjusting for conventional prognostic indicators (Poeta et al. 2007). This study advanced the field and demonstrated that p53 mutational status can be used to sub-classify HNSCC by prognosis. Recently investigators have further identified the significance of disruptive TP53 mutations in lymph node metastasis and overall survival and progression-free survival (Wichmann et al. 2015). Wichmann et al., analyzed data from The Cancer Genome Atlas (TCGA) project and found that lymph node metastases were present in 56% (48 of 86) of the TCGA patients with nondisruptive, and in 69% (58 of 84) of the patients with disruptive TP53 mutations ($p = 0.01$) (The Cancer Genome Atlas 2015; Wichmann et al. 2015).

A new model named the Evolutionary Action score of *TP53*-coding variants (EAp53) was recently put forward to stratify TP53 missense mutations into high-risk and low-risk (Neskey et al. 2015). This new model is an attempt at an improved system to accurately assess outcome of specific mutations. They calculated the evolutionary access scores for TP53 mutations based on the relationship between genotype and phenotype (Katsonis and Lichtarge 2014). In this relationship, the phenotype of a mutation at a certain location in the TP53 gene is determined by the sensitivity of p53 function to variation in said location and the magnitude of the substitution. They calculated the EA score for a multitude of mutations and separated the high-risk versus low-risk group based on an EA threshold score of 75. The investigators found that patients with high-risk TP53 mutations had the worst survival outcomes and were the fastest to develop metastasis (Neskey et al. 2015). They also discovered that tumour cells expressing high-risk TP53 mutations were more invasive, tumorigenic, and more likely to metastasize to the lung (Neskey et al. 2015). This new validated computation model can be considered an extension and elaboration on Poeta et al.’s model of classifying TP53 mutations as disruptive and non-disruptive. Both models supported the finding that HNSCC with mutated p53 have a worse overall survival compared to wild-type p53. In addition, these models support the finding that within the umbrella of TP53 mutants, different TP53 mutations can be stratified based on their prognostic outcome.

5.5 TP53 as a Post-treatment Predictor of Outcome in HNSCC

The treatment of HNSCC requires a multi-treatment approach consistent of radiotherapy, chemotherapy, and surgery. As described by the molecular mechanism in development of HNSCC highlighted above, ionizing radiation can cause DNA damage activating p53 and allowing p53 to regulate cell cycle and apoptosis (Csuka et al. 1997; Perri et al. 2015). In the context of mutated p53, cells become more resistant to radiation-induced cell death (Csuka et al. 1997; Perri et al. 2015). Cells begin to accumulate multiple genetic mutations, which leads to a polyclonal heterogeneous tumour. Tumour heterogeneity has been shown to increase resistance to radiotherapy (Csuka et al. 1997; Perri et al. 2015). In addition, TP53 as predictor of outcome after chemotherapy has also been described. In a prospective series, patients with HNSCC were treated with cisplatin-fluorouracil neoadjuvant chemotherapy (Cabelguenne et al. 2000). TP53 status was characterized in 106 patients with HNSCC and response to chemotherapy was assessed. A correlation was found between presence of mutated p53 and resistance to chemotherapy (Cabelguenne et al. 2000). It is thought that chemotherapy works by inducing programmed cell death in tumor cells, but are unable to do so in the presence of mutated p53 due to impaired p53-associated apoptotic pathway. As a consequence, chemotherapy agents that can induce apoptosis without using the p53 pathway, such as taxanes, were suggested to be effective alternatives to manage the HSNCC population (Perrone et al. 2010).

Since the development of the models by both Poeta et al. and Neskey et al., investigators have tried to use the stratified system of TP53 mutations to study their significance in understanding treatment response. A recent study found that disruptive mutations significantly decrease response to radiation therapy compared to non-disruptive mutations due to inhibition of senescence (Skinner et al. 2012). Another study implemented the EAp53 system and showed that the EAp53 model was able to identify a subset of high-risk patients that had decreased response to platinum based therapy as compared to low-risk patients (Osman et al. 2015). It was also concluded that this model can serve as a predictor for treatment outcome and survival benefit in patients treated with platinum based therapies (Osman et al. 2015). Thus, the stratified classification systems of TP53 mutations and its implications on treatment outcome can serve as an important tool in individualizing and improving treatment for every patient.

5.6 TP53 as a Druggable Target in HNSCC

Given TP53's importance in tumorigenesis as described above and its incidence in HNSCC, many therapeutic strategies have been developed to restore normal p53 function. These therapeutic strategies can be divided into: viral gene therapy to

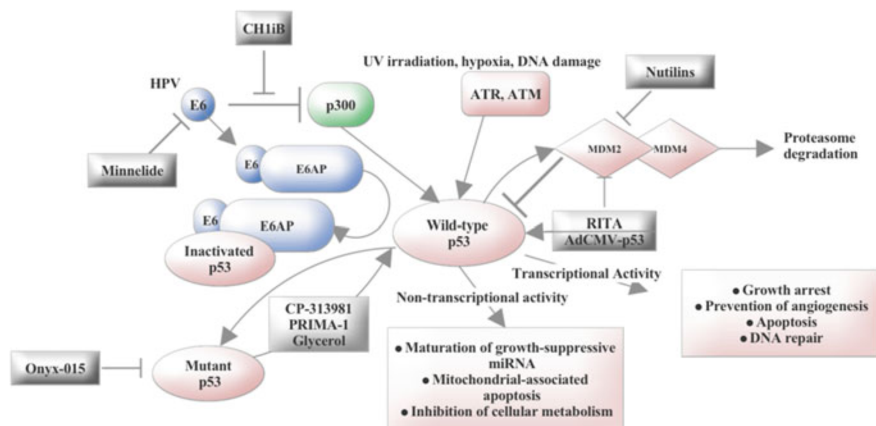

Fig. 5.2 p53-based therapeutics for HNSCC. In normal resting conditions, MDM2-MDM4 heterocomplex targets p53 for ubiquitin-dependent degradation and inhibits its activity. In stressed conditions, the MDM2-MDM4 heterocomplex can undergo degradation leading to activation of p53. Nutlins and RITA restore p53 function by blocking the p53-MDM2 interaction. Stressors such as UV irradiation, hypoxia, and DNA breaks induce activation of p53 program. Activated p53 leads to non-transcriptional and transcriptional downstream effects. AdCMV-p53 increases wild-type p53 levels in HNSCC cells. Onyx-015 selectively eliminates mutant p53 HNSCC cells. CP-31398 stabilizes the DNA binding domain of mutant and wild-type p53. PRIMA-1 restores the ability of mutant p53 to bind to the p53 transcriptional response elements of target genes. Glycerol restores wild-type p53 in mutant p53 HNSCC cells. E6, an HPV oncoprotein, leads to inactivation of wild-type p53 by two different mechanisms. The first mechanism entails E6 binding to E6AP, which leads to ubiquitination of p53 through the proteasome pathway. Minnelide decreases transcription of E6 and reactivates wild-type p53. The second mechanism entails E6 binding to p300, which prevents p300-mediated acetylation and activation of p53. CH1iB disrupts the E6-p300 interaction to reactivate p53 in HPV-positive HNSCC cells

deliver wild-type p53 gene, viruses able to selectively kill cells with nonfunctional-p53, small molecules that act as restoring wild-type function of mutated p53 in tumour cells, and proteins capable of preventing endogenous inactivation of wild-type p53 (Fig. 5.2) (Perri et al. 2015; Tassone et al. 2013).

5.6.1 *Viral Gene Therapy*

The first strategy discussed is the delivery of wild-type p53 through a viral vector, namely adenovirus. Adenovirus was used as the vector to deliver wild-type p53 due to its affinity for cells in the upper digestive tract. AdCMV-p53, a modified adenovirus-5 with CMV promoter and p53 cDNA instead of E1 protein region gene, was used successfully to induce apoptosis in vivo and in vitro (Liu et al. 1995; Nemunaitis and Nemunaitis 2011). This led to a Phase 1 trial using

AdCMV-p53. Thirty-three patients with recurrent HNSCC, 17 of which had non-resectable tumors, and 16 of which had resectable tumors had intratumoral injects of AdCMV-p53 (Clayman et al. 1998). Importantly, no adverse effects were noted and the therapy was well tolerated (Clayman et al. 1998). A subsequent study reported that doctaxel enhanced AdCMV-p53 therapy by increasing viral transduction and increasing expression of exogenously delivered p53, thereby increased apoptosis (Yoo et al. 2004). In 2004, China approved using AdCMV-p53 gene therapy after results showed complete response in 64% of patients in a study of 135 patients (Pearson et al. 2004). After Phase I trials demonstrated the safety of AdCMV-p53 for gene therapy, Phase II trial was conducted on 13 patients with untreated and resectable HNSCC tumors, which showed that 92% of patients were disease free after 1 year. Adverse events were scarce and limited to 3 patients (Yoo et al. 2009). The success of this clinical trial in showing the efficacy and safety of this therapy led to a Phase III trial (Nemunaitis and Nemunaitis 2011). This trial compared AdCMV-p53 to methotrexate in treating recurrent HNSCC. The overall survival between the two treatment arms was statistically insignificant although the median survival in methotrexate treatment arm was slightly improved (7–8 months). It is important to note that the investigators highlighted the classification of favourable and unfavourable biomarkers when assessing overall survival as an outcome. Favourable biomarkers included mutant p53 with low p53 expression and wild-type p53 with either high or low p53 expression and unfavourable biomarkers included mutant p53 with high p53 expression. This further illustrates the need to do an analysis on TP53 mutations to further stratify patients and individualize their treatment based on their specific mutational pattern (Nemunaitis and Nemunaitis 2011).

Given p53's early role in tumorigenesis as highlighted earlier, investigators studied the use of rAdCMV-p53 in oral leukoplakia, a precursor of oral cavity squamous cell carcinoma (Zhang et al. 2009; Li et al. 2009). One study with 18 patients demonstrated a partial or complete resolution of the lesion after intralesional rADCMV-p53 in 72% of patients (Zhang et al. 2009). Another study of 22 patients demonstrated a partial or complete resolution of the lesion in 73% of lesions (Li et al. 2009). In both studies, it was concluded that rAdCMV-p53 was safe in patients with oral leukoplakia, and further clinical trials could highlight the clinical benefit of this gene therapy in oral leukoplakia.

5.6.2 *Selective Killing of Cells with Non-functional p53*

In the above model for gene therapy, the adenovirus vector has the E1 protein region replaced with p53 cDNA. E1 protein region inactivates p53 and allows for cell survival. A lack of E1 protein region renders the virus replication incompetent due to lack of p53 inhibition. On the other hand, a lack of E1 protein region allows viral replication and cell death only in cells with mutant p53 (Perri et al. 2015). This concept was used to formulate oncolytic adenovirus-015 (Onyx-015), a

recombinant adenovirus lacking the E1 protein region, which lyses cells containing mutated p53 (Nemunaitis et al. 2001b). Phase I and II trials have been conducted to assess the efficacy and safety of Onyx-015. A phase I trial assessed the outcome of intratumoral injection of Onyx-015 on 22 patients (Ganly et al. 2000). Twenty-three percent of patients showed improvement in tumor status and the drug was well-tolerated. Another trial was conducted on 10 patients with various malignancies (Nemunaitis et al. 2001a). Two of the patients had HNSCC treated with intravenous Onyx-015. Both patients tolerated the drug well, but neither showed improvement with Onyx-015 (Nemunaitis et al. 2001a). The safety of these trials lead to Phase II trials studying patients with HNSCC treated with intratumoral Onyx-015 or Onyx-015 and standard chemotherapy (Nemunaitis et al. 2001b). In a study using intratumoral Onyx-015 only, 12.5% of patients showed any kind of tumor reduction (Nemunaitis et al. 2001b). In a study using intratumoral Onyx-015 in addition to cisplatin and 5-fluorouracil, 66% of patients showed complete or partial regression, while overall survival was 7–8 months (Lamont et al. 2000). In both studies, Onyx-015 was well tolerated. Although these trials showed only slight therapeutic value in HNSCC, more trials are needed to further elucidate Onyx-015's potential as a therapeutic option.

5.6.3 Restoring Wild-Type Function of Mutated p53

The first molecule to restore wild-type function of mutated p53 is CP-313981. It works by stabilizing the active conformation of the DNA binding domain (Foster et al. 1999). In specific, CP-313981 interacts with newly synthesized p53 mutant protein and changes its conformation to function as wild-type p53 (Rippin et al. 2002). This is further supported by the finding that CP-313981 was unable to refold already mutated protein (Tanner and Barberis 2004). It was also found that CP-313981 plays a role in apoptosis and induces p53 target genes (Takimoto et al. 2002). Specifically, a study of CP-313981 in HNSCC showed that this molecule promotes cell cycle arrest and apoptosis in mutant p53 HNSCC cell lines, but was not as effective in wild-type p53 HNSCC cell lines (Roh et al. 2011). Another molecule, p53 reactivation and induction of massive apoptosis (PRIMA), was discovered through cell-based screening of small molecules. PRIMA-1 was shown to restore conformation of wild-type p53, leading to restoration of p53-dependent transcription in osteosarcoma cell lines (Bykov et al. 2002). Similar to CP-313981, PRIMA-1 was found to promote cell cycle arrest and apoptosis in mutant p53 HNSCC cells lines (Roh et al. 2011). Lastly, glycerol, a chemical chaperone, was found to restore wild-type p53 in mutant p53 HNSCC cell lines (Ohnishi et al. 2000). Glycerol was found to restore heat-induced apoptosis and radiosensitivity in mutant p53 HNSCC cell lines (Ohnishi et al. 2000, 2002). Unfortunately, glycerol is limited in its practical use due to its toxicity (Perri et al. 2015).

A multitude of other molecules such as MIRA-1, chetomin, WR-1065, SCH529074, P53R3, stictic acid, PK7088, zinc metallochapreone-1, STIMA-1,

which are also involved in restoration of wild-type 53 have been studied and their effect documented in other cancers. These molecules have not yet been assessed in HSNCC, but might lead to further promising results in p53 activation in HNSCC (Iwakuma and Parrales 2015).

5.6.4 Prevention of Endogenous Inactivation of p53

As described previously, in normal resting conditions, MDM2 inactivates p53 and stops its function. A whole new class of inhibitors of the MDM2-p53 interaction has been developed. The prototype of this class is nutilins. Nutilins antagonizes MDM2 at the p53-binding interface (Vassilev et al. 2004). Investigators found that nutilin-3 inhibited mutant p53 HNSCC cell lines, but interestingly, inhibited wild-type p53 HNSCC cell lines more effectively (Roh et al. 2011). In these wild-type p53 HNSCC cells, nutilin-3 was able to induce apoptosis through Bax, p21, PUMA in a p53-dependent manner, and to enhance effect of cisplatin (Roh et al. 2011).

Interestingly, a molecule named ‘reactivation of p53 and induction of tumour cell apoptosis’ (RITA) fits in the description of the last two categories. However, it is not clear as to what the exact mechanism by which RITA works. RITA suppresses growth of cancer cell lines by restoration of p53 transcriptional activity and induction of apoptosis (Zhao et al. 2010). In a more recent study it was concluded that RITA induces p53-dependent senescence in HNSCC (Chuang et al. 2014). In addition, RITA was shown to block the interaction between MDM2 and p53 (Issaeva et al. 2004). In the study by Roh et al., it was found that RITA was able to effectively restore p53 function, which induced cell-cycle arrest and apoptosis in mutant p53 HNSCC cell lines and more so in wild-type p53 HNSCC cell lines (Roh et al. 2011). However, it is not clear as to what the exact mechanism by which RITA activates both wild-type and mutant p53 to allow for apoptosis (Iwakuma and Parrales 2015). The combination of RITA and cisplatin was even more efficacious in wild-type HNSCC cell lines.

5.7 p53 in HPV-Positive HNSCC

HPV is a strong risk factor for HNSCC, in particular oropharyngeal cancer (Sturgis and Cinciripini 2007). Epidemiological data indicate that the prevalence of HPV-positive HNSCC has increased by 3–4 fold in the past 3 decades (Sturgis and Cinciripini 2007). In addition, it has been suggested that HPV-positive HNSCC might turn into an epidemic in the near future (Sturgis and Cinciripini 2007). HPV-positive HNSCC usually occurs in patients <60 years of age who are more likely to be non-smokers as compared to patients with HPV-negative HNSCC (Zhou et al. 2016). In HNSCC, HPV 16 in particular, causes more than 90% of

HPV-positive HNSCC (Marur et al. 2010). HPV 16 binds to highly specialized reticulated epithelium especially in the lingual and palatine tonsils (Klussmann et al. 2001). The virus is then able to integrate into the host cell's genome and produce oncoproteins E6 and E7. The E6 oncoprotein is responsible for the degradation of p53 by two different mechanisms. The first mechanism entails E6 binding to E6AP, which acts as an ubiquitin protein ligase in the ubiquitination of p53 through proteasome pathway (Scheffner et al. 1993). The second pathway entails E6 binding to p300, which prevents p300-mediated acetylation and hence activation of p53 (Patel et al. 1999). The E7 protein on the other hand, inactivates pRB, retinoblastoma tumor suppressor gene product, which leads to upregulation of p16 and cell cycle dysregulation (Wiest et al. 2002). Ultimately, both oncoproteins lead to genomic instability and tumorigenesis.

In comparison to HPV-negative HNSCC, of which 78% has TP53 mutations, HPV-positive HNSCC is usually associated with wild-type p53 (The Cancer Genome Atlas 2015). This is supported by the lack of genomic complexity present in HPV-positive HNSCC as compared to HPV negative HNSCC (Agrawal et al. 2011). HPV-positive HNSCCs were found to be dominated by mutations in oncogene *PIK3CA*, novel alterations in*TRAF3*, and amplification of the cell cycle gene *E2F1*. HPV-negative HNSCCs were found to have inactivated *CDKN2A* and amplification of 3q26/28 and 11q13/22 in addition to higher frequency of TP53 mutations (The Cancer Genome Atlas 2015).

HPV-positive HNSCC shows improved sensitivity to chemotherapy and radiotherapy as compared to their HPV-negative counterpart (Ziemann et al. 2015). This increased sensitivity can be due to HPV-positive HNSCC harbouring low levels of normal functioning p53. Radiation therapy causing DNA breaks in tumor cells can activate p53 to induce apoptosis and cell death. Recently the effect of radiation therapy and chemotherapy on HPV-positive HNSCC has been further studied. Investigators found that radiation induces increased expression of E6 and E7 in HPV positive HNSCC (Ziemann et al. 2015). Although an increase in these oncoproteins seems paradoxical, enhanced expression leads to a delay in DNA repair, genomic instability and cell death (Ziemann et al. 2015). Chemotherapy, on the other hand, reduces expression of E6 and E7, leading to less oncogenic activity (Ziemann et al. 2015). This leads to decreased inactivation of p53 and pRb and induction of apoptotic pathways (Ziemann et al. 2015). Therefore, HPV status and p53 status both highlight the difference in radiosensitivity and chemosensitivity in HNSCC leading to different prognostic outcomes after clinical treatment.

Finally, the importance of HPV E6 induced inactivation of p53 is essential to tumourigenesis in HNSCC and therefore a novel arsenal of therapeutics targeting reactivation of p53 may be a valid strategy in HPV-positive HNSCC. There have been many studies looking at therapeutic models targeting E6, E6AP, or the association between E6 and E6AP in cervical cancer (Xie et al. 2014). In HNSCC, investigators demonstrated reactivation of wild-type p53 in HPV-positive HNSCC in vitro and in vivo by triptolide and its prodrug Minnelide (Caicedo-Granados et al. 2014). Tripolide is a diterpenetriepoxide derivative from the Chinese herb *Tripterygium wilfordii*. Tripolide was found to decrease transcription of HPV

oncoprotein E6 and to reactivate wild-type p53, which lead to significant decrease in cell viability and increase in apoptosis in multiple HNSCC cell lines (Caicedo-Granados et al. 2014). Minnelide was also found to decrease tumour volume and progression by activating wild-type p53 (Caicedo-Granados et al. 2014). In addition, the interaction between p53 and p300 has been targeted as a therapeutic strategy. Recently, it was illustrated that CH1iB binds to the CH1 domain on p300 and inhibits the interaction between p53 and p300 (Xie et al. 2014). This inhibitor was found to increase total and acetylated p53 levels, to enhance p53 transcriptional activity, and to increase the expression of p53-regulated genes, p21, miR-34a, and miR-200c (Xie et al. 2014). In addition, CH1iB potentiates the effect of cisplatin (Xie et al. 2014). The discovery of therapeutic targets for p53 reactivation might allow for conservative treatment and may eliminate the need for radical surgery in certain patients.

5.8 Conclusions

The p53 protein is labelled the 'guardian of the genome' due to its function in cell cycle, apoptosis, and a myriad of other cellular processes via transcriptional and non-transcriptional pathways (Lane 1992). TP53 gene mutations occur early and are selectively advantageous in the molecular carcinogenesis of HNSCC. The complexity of TP53 mutations in HNSCC is highlighted by the GOF hypothesis. The GOF hypothesisis evidenced by TP53 mutant's independent oncogenic activities and dominant negative effect on wild-type p53 alleles. The type of mutation and cellular context further illustrate the complexity of GOF activity and mutant TP53 function. The importance of TP53 in the carcinogenesis of HNSCC raises the question of mutant p53's ability to serve as a prognostic biomarker. Although, immunohistochemically, p53's ability to serve as a prognostic biomarker seems lacking in the absence of additional biomarkers, rapid mutational analysis has proven to be much more promising. Rapid mutational analysis led to stratification of p53 mutations into disruptive and non-disruptive (Poeta et al. 2007). Recently, a further classification separated mutations into high-risk and low-risk based on their prognostic implications (Neskey et al. 2015). Currently, studies are identifying the value of these classifications in predicting treatment response. Therefore, stratifying TP53 mutations based on prognostic value and treatment outcome may serve as an important tool in individualizing and improving treatment for every HNSCC patient.

As we understand the importance of TP53 in HNSCC, we raise the need for exploring p53 as a druggable target. Targeting p53 has come in many forms including: viral gene therapy to deliver wild-type p53 gene, viruses selectively killing cells with nonfunctional-p53, small molecules restoring wild-type function of mutated p53 in tumour cells, and proteins capable of preventing endogenous inactivation of wild-type p53 (Perri et al. 2015; Tassone et al. 2013). This new arsenal of therapeutic options will open the avenue for new medications that can be

targeted to individual patients. On the other hand, HPV-positive tumours have a different biological and molecular composition. The E6 oncoprotein degrades p53 and leaves tumour cells with a low level of wild-type p53 leading to tumorigenesis. The wild-type p53 provides improved response to chemotherapy and radiotherapy. Therefore, HPV positivity in HNSCC usually has better prognosis and better treatment outcome. Therapeutic options to reactivate p53 have been a recent area of study in HPV-positive HNSCC. Proteins such as p300 and E6AP, which are involved in degradation of p53 in HPV-positive HNSCC, are targets of some of these therapies.

In conclusion, our understanding of TP53's story in HNSCC has given us insight on the value of TP53 as a prognostic marker, a predictor of clinical treatment outcome, and a druggable target. As a result, TP53 status can be used to provide individualized, customizable, and improved care to patients.

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Chapter 6
Blockage of EGFR Pathway for Anticancer Therapy in Squamous Cell Carcinoma of the Head and Neck

Sandra Schmitz and Jean-Pascal Machiels

Abstract In squamous cell carcinoma of the head and neck (SCCHN), Epidermal Growth Factor Receptor (EGFR) overexpression is linked with poor prognosis. Cetuximab is a chimeric IgG1 monoclonal antibody (mAb) that specifically binds to the EGFR with high affinity. Cetuximab combined with radiotherapy (RT) improves loco-regional control and survival (OS) compared to RT alone in patients with stage III/IV SCCHN, and the addition of cetuximab to 5-fluorouracil and platinum-based chemotherapy improves OS in the first-line treatment of incurable disease. However, the addition of cetuximab to cisplatin-based chemoradiation does not improve progression-free survival (PFS) or OS and only a minority of patients benefits from anti-EGFR mAbs. Other anti-EGFR agents (potentially more potent anti-EGFR mAbs), multiple tyrosine kinase inhibitors (TKIs) and EGFR antisense are currently under investigation. Furthermore, treatment combinations with radio-and or chemotherapy regimens or other monoclonal antibodies have been evaluated and are also under investigation. Efforts to include translational research in all these trials are needed in order to better understand the molecular mechanisms involved, to define molecular criteria for the selection of appropriate patients for targeted therapy and to elucidate the anti-EGFR resistance mechanisms.

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_6

6.1 The Epidermal Growth Factor Receptor (EGFR)

The EGFR is a transmembrane glycoprotein commonly expressed in many normal tissues. It is a member of the HER tyrosine kinase receptor family composed of four different receptors (EGFR/c-erbB-1, c-erbB-2/HER-2/neu, c-erbB-3/HER-3 and c-erbB4/HER-4), all of which are transmembrane proteins with tyrosine kinase activity (Fig. 6.1). The EGFR has an extracellular domain, which provides a ligand-binding site for multiple ligands. Epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and amphiregulin (AR) are specific ligands of the EGFR, while β-cellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR) are less specific ligands that bind EGFR and ErbB4. Upon ligand fixation, EGFR homodimerization or heterodimerization with another HER receptor occurs, leading to the activation of the intracellular tyrosine kinase. This stimulates kinase signal transduction pathways involved in tumor proliferation, apoptosis, angiogenesis and cell migration/invasion (Normanno et al. 2006). Downstream signaling through the Ras/Raf/Mek/Erk pathway controls gene transcription, cell proliferation and cell cycle pro-gression, while the PI3K/protein kinase B (PI3K/Akt) pathway stimulates numerous antiapoptotic signals in the cell (Fig. 1.5). SRC tyrosine

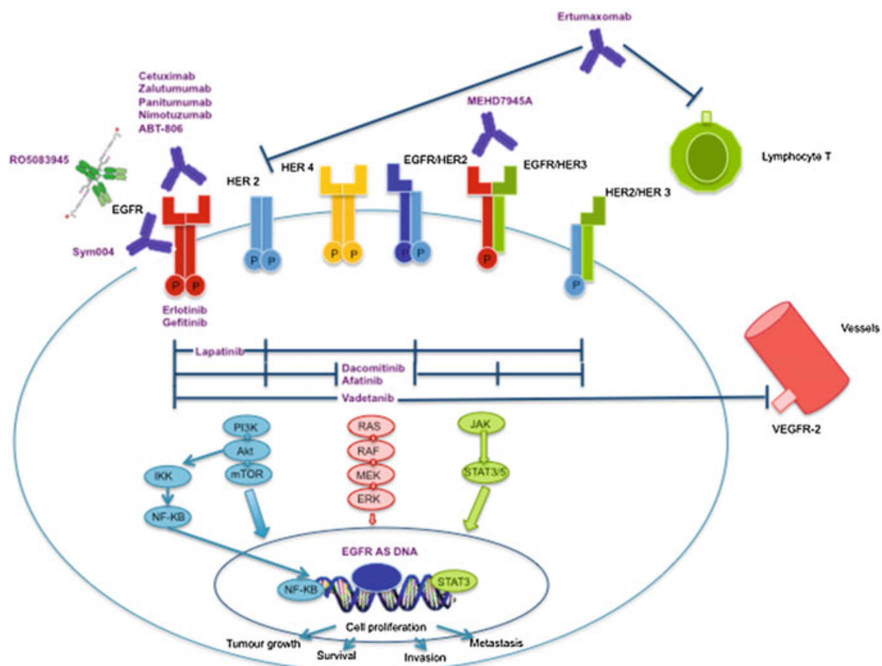

Fig. 6.1 Erb receptors and downstream pathways. Targeted agents tested in clinical trials in SCCHN are indicated in *purple*

kinase, phospholipase-Cγ, protein kinase C (PKC) and signal transducer and activator of transcription (STAT) activation have also been described (Kalyankrishna and Grandis 2006).

Up to 90% of SCCHN express high level of EGFR (Kalyankrishna and Grandis 2006) and overexpression of EGFR and TGF-α are associated with poor prognosis (Ang et al. 2002; Wheeler et al. 2012; Rubin Grandis et al. 1998; Jedlinski et al. 2013) and radioresistance (Ang et al. 2002; Jedlinski et al. 2013; Bentzen et al. 2005). EGFR is upregulated in irradiated cells where it promotes DNA repair and cell cycle arrest in the radioresistant S phase (Dittmann et al. 2005a, b, 2008; Walker et al. 2007). Nuclear localization of the EGFR is correlated with increased expression of cyclin D1, inducible nitric oxide synthase, Aurora A kinase and B-Myb, leading to increased cell cycle progression and proliferation (Hanada et al. 2006; Lo et al. 2005; Hung et al. 2008).

Moreover, Liccardi showed that EGFR nuclear localization was required for repair of cisplatin and radiation-induced DNA damage through association of EGFR with the catalytic subunit of DNA protein kinase (Liccardi et al. 2011). Expression of total and nuclear EGFR is higher in p16-negative tumors compared to p16-positive tumors (Husain et al. 2012), and nuclear EGFR is correlated with poor clinical outcomes in patients with oropharyngeal squamous cell carcinoma (Psyrri et al. 2005). Increased EGFR gene copy number has been reported in 10–58% of patients with SCCHN and has also been described as an indicator of poor survival, loco-regional failure and radioresistance (Temam et al. 2007; Mrhalova et al. 2005; Chung et al. 2006; Chiang et al. 2008). This wide variation in results may be explained by the use of different detection methods (Fluorescence in situ hybridation (FISH) or quantitative PCR) and the molecular heterogeneity of SCCHN.

Mutation analyses are of major interest when analyzing responses to targeted therapies.

In non-small cell lung cancer (NSCLC), EGFR mutations (especially in exon 19 and 20) are correlated with better treatment response to EGFR tyrosine kinase inhibitors (Lynch et al. 2004) and *Kirsten rat sarcoma viral oncogene homolog* (*RAS*) mutations are researched in clinical practice for the treatment of colorectal cancer in order to detect wild type (wt) diseases which are more likely to respond to cetuximab or panitumumab. These mutations are, however, rare in SCCHN. Furthermore acquired resistance through EGFR mutations (T790M) after administration of EGFR TKIs are not documented in SCCHN (D'Arcangelo and Hirsch 2014).

Hama et al. (2009) described four novel mutations (E709K, V765G, Ins770G and G1022S) and one activating mutation of EGFR kinase function [well known in lung cancer (L858R)], in six out of 82 SCCHN samples. E709K, Ins770G and L858R appeared to be functional mutations based on the use of Ba/F3 cells. Of interest, they did not find any mutations in the extracellular domain of EGFR, such as EGFR variant III (EGFRvIII), Other investigators have detected an activating missense mutation (K745R) in 1–2% of SCCHN patients (Loeffler-Ragg et al. 2006; Schwentner et al. 2008).

EGFRvIII is the result of an in-frame deletion of exons 2-7 (deletion of amino acids 30-297, involving 801 base pairs), resulting in a truncated extracellular EGF-binding domain that is constitutively activated (Bigner et al. 1990). The reported frequency of EGFRvIII expression in SCCHN varies from 0 (Lynch et al. 2004) to 42% (Sok et al. 2006; Chau et al. 2011). EGFRvIII preferentially activates the PI3K/AKT pathway, instead of the Ras/Raf/MEK pathway, which is activated by EGFR (Moscatello et al. 1998). EGFRvIII transfected SCCHN cells have increased tumor proliferation in vitro and larger tumor volumes in vivo compared with vector-transfected control cells (Sok et al. 2006). Furthermore, they have less apoptosis in response to cisplatin and less growth inhibition following treatment with cetuximab compared with control cells (Sok et al. 2006). EGFRvIII induces SCCHN cell migration and invasion in conjunction with increased STAT3 activation that cannot be blocked by cetuximab (Wheeler et al. 2010). EGF induces the hypoxia-inducible factor 1-α (HIF1-α) through STAT3. This HIF-1α activation can be abolished by cetuximab in SCCHN cells expressing wild-type EGFR under hypoxic conditions, but not in EGFRvIII-expressing SCCHN cells (Wheeler et al. 2010). Data on the impact of EGFRvIII mutation on response to therapy are scarce and inconsistent. Chau et al. (2011) analyzed tumor specimens of 53 patients treated in 4 phase II trials for recurrent and or metastatic SCCHN with targeted agents. Two trials involved the EGFR inhibitor Erlotinib, whereas the remaining two involved a non-EGFR targeted agent. EGFRvIII mutation, detected in 42% of the study population, appeared to be an unexpected prognostic biomarker associated with better disease control in recurrent or metastatic SCCHN regardless of treatment with erlotinib. In contrast, in another phase II study (Tinhofer et al. 2011) of recurrent or metastatic patients treated with cetuximab and docetaxel, Tinhofer et al. found that patients with EGFRvIII-expressing tumors (17% of the study population) had lower disease control rate (DCR) and shortened PFS (HR: 3.3, $p = 0.005$) but not OS. EGFRvIII mutations were not identified in the 279 HNSCC samples from the Cancer Genome Atlas (TCGA) project (Hayes et al. 2013). Further studies are needed.

6.2 EGFR Inhibition

There are two ways to inhibit EGFR signaling (Fig. 6.1). First, several mAbs administered intravenously can specifically bind to the EGFR with high affinity and thereby block ligand-binding induced receptor activation. These anti-EGFR mAbs also induce EGFR internalization and are responsible for the attenuation of EGFR signaling through their ligands. Second, oral tyrosine kinase competitive inhibitors can reversibly or irreversibly inhibit the binding of adenosine 5'-triphosphate (ATP) to the phosphate-binding loop of the ATP binding site in the intracellular domain of EGFR and thereby abrogate downstream signaling.

6.2.1 Anti-EGFR Monoclonal Antibodies

Cetuximab is a chimeric human/murine IgG1 mAb that specifically binds to EGFR with high affinity. Proposed mechanisms that lead to antitumor activity include the inhibition of receptor activation by natural ligands and endocytosis of the receptor. In addition to this direct effect on the EGFR, cetuximab may also activate several immunologic anti-tumour mechanisms (Torres et al. 2013; Ferris et al. 2010). One of the proposed mechanisms is the binding of the Fc fraction of cetuximab to FcgammaR (FcγR) on natural killer (NK) cells, monocytes, dendritic cells (DC) and other granulocytes, leading to the lysis of cancer cells through antibody-dependent cellular cytotoxicity (ADCC). It has also been postulated that cetuximab may initiate an adaptive immune response via NK cell-induced DC maturation, which enhances cross-presentation to cytolytic T cells (CTL) specific for the EGFR. Elevated circulating EGFR-specific CD8(+) T cells have been found in cetuximab-treated patients with SCCHN (Lee et al. 2011). However, whether ADCC influences cetuximab activity requires further investigations. Finally, cetuximab inhibits DNA double strand-break repair that contributes to resistance to radiotherapy and DNA damage induced by chemotherapeutic agents by preventing nuclear import of EGFR (Chen and Nirodi 2007). This could be one explanation to the synergy observed between EGFR inhibitors and chemotherapy or radiotherapy in preclinical models (Milas et al. 2000).

Although less investigated and characterized, anti-angiogenic properties of cetuximab have also been described in vitro. Luwor et al. found a reduction of HIF-1α and vascular endothelial growth factor (VEGF) expression in response to cetuximab treatment (Luwor et al. 2005). Furthermore, vascular normalization and normalized tumour oxygenation were observed in xenograft models treated by EGFR-TKIs (Cerniglia et al. 2009; Qayum et al. 2009).

Bonner et al. (2006, 2010) conducted a randomized phase III study on 424 patients with locoregionally advanced (stage III or IV) SCCHN in which patients received either radiotherapy alone (213 patients) or radiation plus weekly cetuximab (211 patients). Cetuximab was initiated one week before radiotherapy at a loading dose of 400 mg/m^2, followed by a weekly dose of 250 mg/m^2 during radiotherapy. This trial showed that cetuximab combined with radiotherapy improved median survival from 29.3 to 49.0 months ($p = 0.03$) and locoregional control from 14.9 to 24.4 months ($p = 0.005$). This beneficial effect was mainly seen in the oropharyngeal cancer population. Improvement in overall survival was limited to patients with prominent acneiform rash (hazard ratio 0.49), suggesting its potential role as a predictive marker. However caution should be exercised in stressing this point as today we could treat acneiform rashes more early and aggressively. Human papilloma virus (HPV) status was retrospectively assessed in this trial and univariate analyses showed a more pronounced treatment effect of radiotherapy and cetuximab versus radiotherapy in p16+ patients across all endpoints in both the intent-to-treat (ITT) and the oropharyngeal populations. However, interaction tests for locoregional control (LRC), OS and PFS in both

populations did not demonstrate a significant interaction between p16 status and treatment effect (Rosenthal et al. 2014). The Radiation Therapy Oncology Group (RTOG) 1016 (NCT01302834) trial is ongoing and tries to respond specifically to this question by evaluating the radiosensitizing effect of cetuximab versus cisplatin in HPV positive oropharyngeal SCC. So far, in locally advanced (LA) SCCHN, the combination of radiation therapy and cetuximab has never been compared to the more validated standard treatment, which is platinum-based chemoradiation (Pignon et al. 2000). Two studies are currently ongoing: a phase II trial comparing cetuximab plus radiotherapy versus cisplatin plus radiotherapy in LA SCCHN (NCT01216020) and a phase III trial comparing radiotherapy plus cetuximab versus concurrent chemoradiotherapy in HPV associated oropharynx cancer (NCT01302834).

The RTOG conducted a phase III trial (RTOG 0522) investigating the addition of cetuximab to the platinum-based chemoradiation (Ang et al. 2011). Patients with stage III/IV SCCHN were randomized to receive accelerated radiation and concurrent cisplatin with or without weekly cetuximab. No difference was observed between the two groups with regards to PFS and OS. The 2-year PFS in the chemoradiation group was 64.3%, while in the chemoradiation group plus cetuximab it was 63.4% with a hazard ratio (HR) of 1.05 ($p = 0.67$). The 2-year OS was 79.7 and 82.6% respectively, with a HR of 0.87 ($p = 0.17$). However, local toxicity was higher in the cetuximab-containing arm, with grade 3 and 4 stomatitis being observed in 43% versus 33% and in-field dermatitis in 25% versus 15%. In contrast, the rate of toxic deaths was not increased, being 2% in both arms (Ang et al. 2002). Recently Weidhaas et al. investigated retrospectively the impact of the *KRAS*-variant in the RTOG 0522 study. The *KRAS*-variant is a germ-line mutation in a microRNA-binding site in *KRAS*. They found that patients with the *KRAS*-variant appeared to better respond to cetuximab, increasing PFS and OS compared with patients without the *KRAS*-variant (Weidhaas et al. 2014).

Lefebvre et al. recently reported the results of a phase II trial comparing the efficacy and safety of induction chemotherapy (3 cycles of docetaxel and cisplatin, 75 mg/m^2 for both agents on day 1 and 5-fluorouracil 750 mg/m^2 per day from days 1 through 5) followed by concurrent regimens for larynx preservation (TREMPLIN). Poor responders to induction chemotherapy (<50% response) underwent salvage surgery. Responders were randomly assigned to conventional radiotherapy plus either cisplatin or cetuximab. Primary end point was 3-month larynx preservation. Of the 153 enrolled patients, 116 were randomly assigned after induction chemotherapy. Overall toxicity of both chemoradiation and radiotherapy plus cetuximab regimens was substantial following induction chemotherapy. In an intent-to-treat analysis, there was no significant difference between the arms in the rates of larynx preservation at 3 months and OS at 18 months (Lefebvre et al. 2013). Treatment compliance was higher in the cetuximab plus radiation therapy arm.

Ghi et al. randomized patients with unresectable stage III-IV SCCHN according to a 2 $\times$ 2 factorial design: 2 cycles of cisplatin/5-fluorouracil concomitant to radiotherapy (arm A1), cetuximab concomitant to radiotherapy (arm A2), 3 cycles

of induction cisplatin, docetaxel, and 5-fluorouracil (TPF) followed by concurrent platinum-based chemoradiation (arm B1) and 3 cycles of TPF followed by cetuximab and radiotherapy (arm B2) (Ghi et al. 2013). A total of 421 patients were randomized: 261 received concomitant chemoradiation and 160 concurrent cetuximab and radiotherapy. Interestingly, no significant differences for grade 3 and 4 in-field skin and mucositis toxicities were observed, challenging the concept that cetuximab added to radiation therapy is less toxic than cisplatin-based chemoradiation. In addition, compliance to concomitant treatment was more favorable in the chemoradiation arm in terms of systemic treatment completion ($p = 0.005$), treatment duration ($p < 0.001$) and unplanned interruptions of radiotherapy for ≥ 3 consecutive days ($p = 0.001$). Both regimens showed similar efficacy: median PFS and OS were 21.6 months and 44.7 months for the chemoradiation arm and 20.7 months and 44.7 months for the cetuximab and radiotherapy arm. However, this study has not enough power to demonstrate that cetuximab plus radiation therapy is equivalent to cisplatin-based chemoradiation. Recently the same group presented the results of the comparison between induction and non induction arms and concluded that Induction TPF followed by chemoradiation or cetuximab plus radiotherapy significantly improved PFS and OS (53 months vs. 30 months, $p = 0.015$; independently from the type of concomitant strategy) patients without compromising compliance to the concomitant treatments (Ghi et al. 2014). However, the benefit of induction in the context of platinum-based concomitant chemoradiation was not clearly demonstrated in the Ghi's study. Further trials to answer these questions are therefore needed. NCT00999700 trial compares concurrent chemoradiation regimen to induction chemotherapy followed by radiotherapy and cetuximab and NCT00716391 trial compares induction chemotherapy followed by radiotherapy and cetuximab with induction chemotherapy followed by platinum-based chemoradiation.

Cetuximab has also been evaluated as monotherapy or in combination with chemotherapy in patients with R/M platinum-refractory SCCHN. The results of 2 phase II trials investigating the addition of cetuximab to carboplatin or cisplatin in patients with platinum-refractory SCCHN showed an objective response rate (ORR) of 10% and a median survival of 5.2–6.1 months (Baselga et al. 2005; Herbst et al. 2005). Another phase II study with similar inclusion criteria evaluated cetuximab as monotherapy and reported an ORR of 13% with a median OS of 5.9 months (Vermorken et al. 2007). This last study suggests that single agent cetuximab, in this platinum-refractory population, can offer similar results to those obtained when the drug is used in combination with a platinum compound. A pooled analysis of these three phase II trials (n = 278) (Vermorken et al. 2008a) was performed and the results were compared to those from Leon's retrospective study in which patients (n = 151) (Leon et al. 2005) were treated with best supportive care (BSC) or various second-line treatments. This indirect comparison suggests that cetuximab has the potential to increase median OS by approximately two months. Although a randomized trial is missing in the recurrent, metastatic or palliative setting, cetuximab was approved by the Food and Drug Administration

(FDA) as monotherapy in R/M disease after platinum-based treatment (www.fda.gov).

Burtness et al. (2005) conducted a randomized trial (ECOG E5397) comparing cisplatin (100 mg/m^2 every 4 weeks) and placebo to cisplatin and cetuximab (400 mg/m^2 loading dose followed by 250 mg/m^2 weekly) in chemotherapy-naïve SCCHN patients with incurable SCCHN. There was a statistically significant higher response rate in the cetuximab arm, 26% versus 10% ($p = 0.03$), but no significant difference in PFS (the primary endpoint) or OS.

The phase III EXTREME trial (Vermorken et al. 2008b) randomized 442 patients with recurrent or metastatic SCCHN to receive 5-fluorouracil (5-FU) and platinum based therapy alone or in combination with cetuximab as a first-line palliative regimen. In the experimental arm, patients who had at least stable disease (SD) after a maximum of six cycles of chemotherapy received cetuximab monotherapy until the disease progressed or occurrence of unacceptable toxic effects. This study demonstrated a significant benefit of cetuximab with an improvement in median OS from 7.4 months to 10.1 months ($p = 0.04$). Another important finding of the EXTREME trial was the lack of increase of chemotherapy-associate toxicities in the cetuximab-containing arm. However, significantly more patients in the cetuximab arm experienced sepsis (9 vs. 1; $p = 0.02$). An unplanned retrospective analysis assessed the outcome by tumor p16 status (as a surrogate marker for HPV) and concluded that the survival benefit of adding cetuximab to chemotherapy was independent of tumor p16 status, and that patients with p16-positive tumors had a more favorable outcome than those with p16-negative tumors. However, this last analysis is limited by the low number of patients with p16+ disease (Vermorken et al. 2014).

More recently Cetuximab was investigated in a window of opportunity studies in operable treatment naïve patients with SCCHN. The authors conclude that short course pre-operative administration of cetuximab was safe. The high rate of ^{18}FDG-PET response was correlated with reduced tumour cellularity in the surgical specimen suggesting the potential role of ^{18}FDG-PET as early marker of cetuximab activity in SCCHN (Schmitz et al. 2013).

Zalutumumab is a fully human IgG1 monoclonal antibody which targets EGFR. Machiels et al. (2011) randomized 286 patients with recurrent and/or metastatic SCCHN that progressed within 6 months of platinum-based therapy to receive either zalutumumab plus BSC or BSC with optional methotrexate, in a 2:1 ratio. Zalutumumab was administered at a loading dose of 8 mg/kg followed by increasing titrated doses of 4 mg/kg every 2 weeks until the appearance of a grade 2 rash. This study did not meet its primary endpoint of improving OS (median: 6.7 months in the zalutumumab group vs. 5.2 months in the control group; $p = 0.06$). However, PFS was significantly higher in the zalutumumab group ($p = 0.001$) suggesting some clinical activity of this agent.

Panitumumab is a fully human IgG2 mAb against EGFR. The SPECTRUM trial (Vermorken et al. 2013) randomized 657 patients with recurrent/and/or metastatic SCCHN cisplatin and 5-FU alone or with panitumumab (9 mg/kg), every 3 weeks. Panitumumab did not significantly improve the OS (median: 11.1 months vs.

9.0 months, $p = 0.14$), the primary endpoint, but did yield significantly higher ORR (36% vs. 25%; $p = 0.007$) and PFS (5.8 months vs. 4.6 months; $p = 0.004$). Recently, the authors presented (Stoehlmacher-Williams et al. 2012) an unplanned analysis of the results stratified by tumor p16 status, which suggested that panitumumab improved OS and PFS in patients with p16-negative tumors but not in those with p16-positive. These results should be interpreted with caution because of the low number of patients with p16 positive tumors as well as the definition of p16 positivity (more than 10% of the tumor cells expressing p16 in contrast to the more commonly accepted criterion of more than 70%). In addition, as mentioned above, a similar analysis in the EXTREME study, using the 70% cut-off point, did not show the same results (Psyrri et al. 2012). Therefore, further evaluation is needed to better understand the impact of HPV and/or p16 status on the activity of anti-EGFR mAbs.

The phase II CONCERT-1 study showed that the addition of panitumumab to cisplatin-based chemoradiation resulted in increased toxicity with no improvement in response rate or survival. There was also no difference in efficacy when patients were stratified according to the tumor p16 status (Giralt et al. 2012a). In the CONCERT-2 trial, the investigators compared the safety and efficacy of radiation therapy combined with panitumumab without chemotherapy versus concurrent chemoradiation. The investigators reported a trend in favour of concurrent chemoradiation for locoregional control at 2 years (the primary endpoint); 51% (40–62%) with irradiation plus panitumumab compared to 61% (47–72%) with concurrent chemoradiation. Both PFS (HR = 1.73, $p = 0.03$) and OS (HR = 1.59, $p = 0.10$) outcomes favored the concurrent chemoradiation arm (Giralt et al. 2012b). A randomized (1:1) phase II study investigated docetaxel and cisplatin with or without panitumumab in the first-line palliative treatment (Wirth et al. 2013). Median PFS, median OS and overall response rate were 6.9 months, 12.9 months and 44% in the panitumumab arm versus 5.5 months, 13.8 months and 37% in the arm without panitumumab. Crossover to panitumumab monotherapy was allowed in the docetaxel/cisplatin arm and occurred in 57% of the patients and may have impaired the OS analysis. Panitumumab is currently being tested in different indications, either in monotherapy or in combination with chemotherapy (NCT00446446, NCT00454779) in the recurrent and/or metastatic setting, in combination with chemoradiation for LA disease or in a window of opportunity study (NCT01305772) in order to determine gene signature profiling and metabolic modifications.

Nimotuzumab (h-R3mAb) is another monoclonal antibody that bivalently binds to the extracellular domain of the EGFR that overlaps with the binding site of cetuximab and EGF. Different phase II studies also investigated this agent in combination either with radiation or chemoradiation in advanced SCCHN. The randomized phase II trial of Rodriguez et al. (2010) compared radiotherapy and nimotuzumab to radiotherapy and placebo in a group of 106 patients with unresectable SCCHN who were unfit for concurrent chemoradiation. Toxicities were limited to almost only grade I/II. The complete response rate was significantly higher in the nimotuzumab group (59.5%) versus the placebo group (34.2%) ($p = 0.038$), as was survival ($p = 0.0491$). However, the radiation dose used in this

study was low and could be questioned. Another phase II study by Babu et al. (2010) randomized 92 patients with locally advanced (LA) SCCHN to receive radiotherapy alone or radiotherapy plus nimotuzumab (arm A) or chemoradiation without or with nimotuzumab (arm B). Locoregional response was better in the nimotuzumab arms: 76% versus 37% in arm A, and 100% versus 70% in arm B. The corresponding OS rates at 48 months were 34% versus 13% (non-significant) in group A, and 47% versus 21% in group B ($p = 0.01$). The addition of nimotuzumab to chemoradiation resulted in a significant reduction in the risk of death by 65% (HR 0.35, $p = 0.01$). These results should, however, be interpreted with caution due to the low number of patients included. Other ongoing nimotuzumab studies are addressing its combination with radiation or chemoradiation (NCT00702481, NCT00910117), with postoperative adjuvant chemoradiation (NCT00957086), or with neoadjuvant chemotherapy (NCT01516996).

ABT-806 is a humanized IgG1 monoclonal antibody targeting an unique EGFR epitope exposed only when EGFR is overexpressed or mutated (EGFRvIII). A phase 1 study of ABT-806 included 26 patients (2–24 mg/kg/every other week) and showed encouraging results: 5 patients, including two with SCCHN, achieved SD for $\geq$8 weeks. One patient with *EGFR* amplified SCCHN had a stable SD for 23 weeks. No patient had a typical EGFR inhibitor-induced rash (Cleary et al. 2012). A recent phase I study also described a high therapeutic index and specificity of ABT-806, supporting further investigation of this treatment either as monotherapy or as an antibody-drug conjugate. Among 18 patients with advanced tumors likely to express EGFR, 1 patient with SCCHN had a confirmed partial response (PR) and 5 other patients had SD (lasting 37 and 24 weeks in a patient with adrenal carcinoma and SCCHN, respectively) (Gan et al. 2013).

Finally, one phase I trial (Lai et al. 2009) investigated the safety of intratumoral injection of **EGFR antisense** (AS) DNA, which is an EGFR antisense gene sequence under the U6 promoter control. The EGFR AS was given in four weekly injections and the dose was escalated in consecutive cohorts (six dose levels; 60–1920 μg/injection). The maximum tolerated dose was not reached as no grade 3 or 4 dose-limiting toxicities were noted in the 17 patients studied. Five patients (29%) had objective responses (two complete and three partial responses). Two patients had SD. Patients with disease control (CR + PR + SD) had tumors with higher EGFR and lower STAT3 expression at baseline compared to patients who had progressive disease ($p = 0.03$ and $p = 0.01$, respectively). A phase I/II study is currently investigating the combination of intratumoral EGFR AS, radiotherapy and cetuximab in patients with LA SCCHN who are elderly or not eligible for cisplatin (NCT00903461).

6.2.2 EGFR Tyrosine Kinase Inhibitors (TKI)

Reversible “selective” EGFR TKIs, such as gefitinib and erlotinib, have been investigated in SCCHN.

A phase II **erlotinib** trial (initial dose 150 mg/day) yielded 5 PRs in 115 patients with recurrent and/or metastatic SCCHN (Soulières et al. 2004). Disease stabilization was maintained in 44 patients (38.3%) for a median duration of 16.1 weeks. The median PFS was 9.6 weeks and the median OS was 6.0 months. Rash and diarrhea were the most common drug related toxicities, encountered in 79 and 37% of patients, respectively, though most were mild. Subgroup analyses revealed a significant difference in OS favoring patients who developed grade ≥ 2 rash compared to those who did not ($p = 0.045$). No difference in survival was detected based on HER1/EGFR expression. Erlotinib was also investigated in a preoperative window study: Thomas and colleagues conducted a phase II pre-operative study with erlotinib given for a median of 20 days (range: 18–30). They concluded that baseline p21waf expression was associated with response to erlotinib and identified cyclin-dependent kinase 2-interacting protein as a potential marker of erlotinib efficacy (Thomas et al. 2013). Another group investigated erlotinib and erlotinib plus sulindac, a non-selective COX inhibitor in a randomized, placebo-controlled pre-operative window trial. Although limited by the low number of patients, their data suggested additive antiproliferative effects (KI67) of the two agents and identified pSrc as a potential resistance biomarker of anti-EGFR therapy (Gross et al. 2014).

Single-agent **gefitinib** (ZD1839) at a dose of 500 mg/day was investigated in a phase II trial in 52 patients with recurrent or metastatic SCCHN (Cohen et al. 2003). Half the cohort received gefitinib as second-line therapy. Forty-seven patients were assessable for response, with an observed response rate of 10.6% and a disease control rate of 53%. The median time to progression and survival were 3.4 and 8.1 months, respectively. The only grade 3 toxicity encountered was diarrhea in three patients. Performance status and development of rash were found to be strong predictors of response, progression, and survival. Ten paired biopsy samples were assessable and revealed no significant change in EGFR or p-ERK expression with this treatment (Thomas et al. 2007). A phase III trial (IMEX) compared methotrexate alone to gefitinib alone as palliative therapy for incurable, recurrent SCCHN (Stewart et al. 2009). Four hundred eighty-six patients were randomly assigned to oral gefitinib at 250 mg/day, oral gefitinib at 500 mg/day, or weekly intravenous methotrexate at 40 mg/m^2. No significant differences between either gefitinib regimen and methotrexate were found. The median OS were 5.6, 6.0, and 6.7 months and the ORR were 2.7, 7.6 and 3.9%, respectively. No unexpected adverse events (AE) were observed, except for more tumor hemorrhage events in the gefitinib arms (8.9% in gefitinib 250 mg/day; 11.4% in gefitinib 500 mg/day, and 1.9% in methotrexate).

In another phase III trial (Argiris et al. 2013), 270 patients were randomly assigned to receive docetaxel 35 mg/m^2 IV on days 1, 8, and 15, every 28 days plus placebo (arm A) or plus daily gefitinib 250 mg given orally (arm B) until disease progression. The study was stopped at the interim analysis in November 2008 because of the low likelihood of meeting the primary endpoint (increase in OS from 6.0 to 8.4 months). Median OS was 6 months in arm A versus 7.3 months in arm B

($p = 0.60$). Median time to progression (TTP) was 2.1 months and 3.5 months ($p = 0.19$), respectively. An unplanned subset analysis showed that gefitinib improved survival in patients younger than 65 years (median OS: 7.6 months vs. 5.2 months; $p = 0.04$). There was also improvement in TTP in arm B for younger patients (median TTP 3.6 months vs. 2.0 months, $p = 0.01$) but not for patients ≥ 65 years (median TTP 4.4 months vs. 3.7 months; $p = 0.58$). Also, there was a trend for improved survival in patients with wild-type c-MET tumor (5.7 months vs. 3.6 months; $p = 0.09$) regardless of treatment. Grade 3/4 toxicities were comparable between the two arms except for grade 3/4 diarrhea, which was more common in the docetaxel/gefitinib group.

Some investigators have attempted to add EGFR TKIs to chemoradiation in LA SCCHN. Martins et al. (2013) randomized 204 patients in a phase II study with LA SCCHN to receive radiotherapy and cisplatin 100 mg/m^2 (days 1, 22, 43) without (arm A) or with 150 mg of erlotinib (arm B) starting one week before and during chemoradiation. The most common serious adverse events (SAE) were nausea, vomiting, and dehydration accounting for 30% of all serious adverse event reported in both arms. Arm A had a CRR of 40% and arm B had a CRR of 52% ($p = 0.08$). With a median follow-up of 26 months and 54 progression events, there was no difference in PFS (hazard ratio, 0.9; $p = 0.71$). In a randomized phase II trial, Gregoire et al. (2011) enrolled 226 patients to receive either gefitinib 250, 500 mg/day or placebo in two phases: a concomitant phase (gefitinib or placebo with chemoradiotherapy), followed by a maintenance phase (gefitinib or placebo alone). The investigators concluded that gefitinib (250 and 500 mg/day) did not improve the 2-year local DCR compared with placebo, when given either concomitantly with chemoradiation (32.7% vs. 33.6%, respectively, $p = 0.607$) or as maintenance therapy (28.8% vs. 37.4%, $p = 0.894$).

6.3 Strategies to Overcome Resistance to Anti-EGFR Therapy

6.3.1 Blockage of Multiple HER Receptors

Beside EGFR, C-erbB-2/HER-2-neu, c-erbB-3/HER-3 and c-erbB4/HER-4 are other members of the HER tyrosine kinase receptor family.

The *HER-2/neu* gene encodes a transmembrane protein of 185 kDa. HER-2 has no ligand, but the intracellular part of this receptor has tyrosine kinase activity. It can dimerize spontaneously or form heterodimers with other members of the EGFR family to activate some of the downstream signal-transduction pathways implicated in carcinogenesis. HER-3 does not have intrinsic tyro-sine kinase activity but can be transphosphorylated by EGFR and HER-2/neu. HER-4, the fourth member of the family, encodes a 180-kDa transmembrane tyrosine kinase that can also form heterodimers with the other HER receptors.

The overexpression rates of HER-2, HER-3 and HER-4 in SCCHN have been reported by different groups. However, conflicting results exist and their value as a prognostic tool is still unclear. For example, HER-2 is overexpressed in 5–33% of patients with SCCHN, but contrary to gastric and breast carcinomas, where HER-2 overexpression or amplification is linked to a poorer prognosis and a decrease in OS, results in SCCHN are conflicting (Del Sordo et al. 2010; Cavalot et al. 2007). This is probably due to the different methods used to detect the protein. Even if some studies have suggested that HER-2 overexpression may correlate with poorer DFS, larger studies using standardized methodology are needed to fully assess its prognostic significance in SCCHN (Cavalot et al. 2007; Ekberg et al. 2005). Some studies have also shown that HER-3 could have a significant impact on survival (Del Sordo et al. 2010). Wilson et al. identified, particularly in cells derived from SCCHN, a subset of non-HER2 amplified cancer cells, with sensitivity to HER2 kinase inhibition. They explained this observation by activation of HER3 by a neuregulin-1 (NRG1)-mediated autocrine loop and concluded that patients with NRG1-driven tumors lacking HER2 amplification may derive significant clinical benefit from HER2/HER3-directed therapies (Wilson et al. 2011). Recently Saba et al. investigated the expression of EGFR, HER-2 and HER-3 in oropharynx SCC and concluded that HER3 may play a role in HPV negative patients with low EGFR levels (Saba et al. 2014). The role of HER-4 has not been widely studied. Heterodimerization of EGFR with HER-2, HER-3 or HER-4 induced by ligand binding may be responsible for the limited activity of EGFR targeted mAbs or TKIs. The ability to block more than just one HER receptors could therefore be of interest. Furthermore, resistance mechanisms through activation of various other receptors and intracellular pathways have been described, including the insulin growth factor receptor-1 (IGF-1R), the hepatocyte growth factor receptor (c-Met), the PI3K/Akt pathway, the STAT pathway and modifications of vascularization and interaction with head shock proteins.

Lapatinib is an oral reversible dual tyrosine kinase inhibitor of EGFR and HER2. Del Campo et al. (2011) investigated the effect of lapatinib alone in previously untreated LA SCCHN by randomizing (2:1) 107 patients to receive lapatinib or placebo for 2–6 weeks before cisplatin-based chemoradiation therapy. The objective response rate (ORR) in 40 patients that received >4 weeks of lapatinib was 17%. Examination of biopsy specimens showed that lapatinib monotherapy decreased the proliferation index Ki67 ($p = 0.03$) without increasing apoptosis. There was no significant difference in ORR between lapatinib (70%) and placebo (53%) arms after completion of chemoradiation. Adding lapatinib to TPF induction chemotherapy followed by chemoradiation was found to be too toxic. Of the 7 patients with LA larynx or hypopharynx SCC enrolled, 4 experienced DLTs at the first dose level of lapatinib. Therefore, the trial was terminated (Lalami et al. 2012).

Harrington et al. (2013) treated 67 patients with LA SCCHN with either daily lapatinib 1500 mg or placebo plus 3 cycles of cisplatin, 100 mg/m^2 (days 1, 22, 43), and radiotherapy (70 Gy/35 fractions/7 weeks) followed by maintenance lapatinib/placebo. This study showed that lapatinib was well tolerated when combined with chemoradiation. The complete response rates at 6 months were 53%

with lapatinib and 36% with placebo. The PFS and OS rates at 18 months were 55% versus 41% and 68% versus 57%, respectively. The difference between study arms was greatest for patient with p16-negative tumors: The median PFS was >20.4 months with lapatinib versus 10.9 with placebo. The HR for p16-negative lapatinib versus p16-negative placebo was 0.556. The same investigator presented recently the results of a study (NCT00424255) combining lapatinib to chemoradiation in high risk patients. They did not observe significant differences in DFS for any of the pre-specified subgroups (nodal status, primary tumor location, geographical region and ErbB1 expression), including HPV (Harrington et al. 2014). Other studies combining lapatinib to chemoradiation are ongoing (NCT01711658, NCT00387127). NCT00490061 addresses the combination of lapatinib with radiotherapy in patients with LA SCCHN unable to tolerate chemotherapy.

Lapatinib used in the setting of recurrent and/or metastatic SCCHN yielded no ORR (de Souza et al. 2012) in either EGFR inhibitor naive or refractory patients.

More recently, a new generation of HER inhibitors, the irreversible small molecule pan-HER inhibitors including **afatinib** and **dacomitinib**, has been developed. By covalently binding and irreversibly blocking all kinase receptors from the ErbB family, a prolonged inhibition is obtained with the aim of improving clinical activity.

Afatinib is a TKI that irreversibly inhibits EGFR, HER-2, and HER-4 kinases. Seiwert et al. (2014) randomized 124 patients with metastatic or recurrent SCCHN that failed platinum-containing therapy to receive 50 mg of afatinib daily or cetuximab (400 mg/m^2 initial loading dose). According to independent central review, ORRs were 8.1% for afatinib and 9.7% for cetuximab. Median PFS times were 13 and 15 weeks in the afatinib and cetuximab arms, respectively. Cross-over to the other treatment was allowed after tumor progression or drug related AE and occurred in 56% of the patients. For the 32 patients crossing from afatinib to cetuximab, the DCR was 19% and the median PFS 5.7 weeks whereas for the 36 patients switching from cetuximab to afatinib, the DCR was 33% and the median PFS 9.3 weeks, supporting the hypothesis that sequential therapy with afatinib may have activity in patients pretreated with cetuximab. The phase III trial LUX-Head & Neck 1 study (NCT01345682) and LUX Head & Neck 3 study (NCT01856478) are currently investigating afatinib versus methotrexate in recurrent and/or metastatic SCCHN patients following progression after platinum-based chemotherapy. Furthermore a study evaluating afatinib versus placebo as adjuvant therapy following treatment by chemoradiation for LA disease (NCT01345669/LUX-Head and Neck 2) is ongoing as well as one study where afatinib is tested as maintenance therapy after postoperative CRT (NCT01427478). Two window of opportunity studies are also recruiting patients to evaluate pharmacodynamic markers after afatinib administration in previously untreated patients with SCCHN (NCT01415674 and NCT01538381).

Dacomitinib (PF00299804) is an irreversible, small molecule inhibitor of EGFR, HER-2 and HER-4 tyrosine kinases. A recently published phase II study of 69 patients with recurrent or metastatic SCCHN showed a PR rate of 12.7% and SD of 57.1%, which lasted for ≥ 24 weeks in 14.3% of patients. The median PFS was

12.1 weeks and the median OS was 34.6 weeks. The most common grade 3–4 AEs were diarrhea (15.9%), acneiform dermatitis (8.7%), and fatigue (8.7%) (Abdul Razak et al. 2013). Furthermore, a window preoperative study with administration of Dacomitinib is ongoing (NCT01116843).

Dual targeting mAbs or mixture of mAbs

A large variety of bispecific antibodies are under development. The goal of such antibodies is to simultaneously block two receptors or mediators in order to overcome resistance or to stimulate immune effector cells. Acquired resistance to anti-EGFR treatment through ERBB2 amplification or via the upregulation of heregulin, a ligand of ERBB3 and ERBB4, has been reported (Yonesaka et al. 2011). Therefore, inhibition of ERBB2 or disruption of ERBB2/ERBB3 heterodimerization could restore cetuximab sensitivity in vitro and in vivo (Baselga and Swain 2009; Wilson et al. 2011).

The IgG1 antibody **MEHD7945A** inhibits both EGFR- and HER3-mediated signaling and mediates ADCC in vitro and in vivo (Schaefer et al. 2011). A phase I study of 36 patients showed an encouraging safety profile and evidence of anti-tumor activity when given at a dose of 14 mg/kg every 2 weeks (Cervantes-Ruiperez et al. 2009). Common grade 1–2 AEs included rash/dermatitis (53%), diarrhea (36%), fatigue (22%), paronychia (19%), dry skin, nausea and decreased appetite (cumulative 17%), asthenia, and stomatitis/oral pain (cumulative 14%) but no grade 3–4 AEs were observed. Best response by RECIST criteria included two PRs (both SCCHN) and 6 SD $\geq$ 8 weeks (2 non small cell lung cancer and 4 colorectal cancer). Seven out of eight patients were previously treated with EGFR inhibitors. An ongoing randomized phase II trial (NCT01577173) is comparing MEHD7945A against cetuximab in patients with recurrent/metastatic SCCHN that progressed during or shortly after platinum-based chemotherapy.

Other investigators aim to stimulate immune effector cells and to enhance receptor specific effects. Schroeder et al. (2011) evaluated the potency of **catumaxomab** (anti-EpCAM $\times$ anti-CD3) and **ertumaxomab** (anti-HER-2/neu $\times$ anti-CD3) with a cytotoxicity assay using mononuclear cells isolated from the samples of 12 patients with SCCHN (who had undergone treatment with 5-fluorouracil, cisplatin and radiotherapy), in co-culture with a tumor target cell line. The study showed that most patients had decreased immune cell counts during the course of chemotherapy. Nonetheless, an effective and concentration-dependent anti-tumor activity, mediated by trifunctional antibodies, was demonstrated using these patient immune effector cells.

Finally, **Sym004** is a drug mixture of two IgG1 mAbs targeting non-overlapping epitopes on the EGFR. In preclinical models, Sym004 exhibited more pronounced EGFR internalization, degradation and tumor growth inhibition than cetuximab (Pedersen et al., 2010). Sym004 was recently investigated as palliative monotherapy in patients with SCCHN (Machiels et al., 2013). Twenty-six patients, of whom 23 (88%) had progressed while on anti-EGFR mAb treatment, were included. Median PFS and OS were 82 days and 156 days, respectively. No objective responses were recorded and 13 (50%) patients had SD as best response. Minor tumour shrinkages were observed in 8 (31%) patients.

RO5083945 is a glycoengineered human monoclonal antibody antagonist of EGFR that seems to exhibit increased binding affinity for all FcγRIIIa variants expressed on immune effector cells. An exploratory, phase I is comparing the pharmacodynamics of **RO5083945** to cetuximab in patients with operable SCCHN.

6.4 Combinations of Anti-EGFR Therapy with Other Molecular Pathways Inhibitors

6.4.1 IGF-1R Inhibitors

The IGF-1 receptor (IGF-1R) is a transmembrane heterotetramer receptor that consists of two α and β two subunits. Its ligands are both IGF-1 and IGF-2. After ligand binding to IGF-1R, two major downstream signaling cascades are activated: the PI3K/Akt/mTOR pathway and the Ras/Raf/MAPK path-way. The IGF-1R plays an important role in cell growth, proliferation, cell differentiation, antiapoptotic signaling and neovascularization (Kurihara et al. 2000; Tang et al. 2003; Resnicoff et al. 1995). This receptor is overexpressed in 73% of SCCHN (Baserga 2005; Barnes et al. 2007; Jun et al. 2009) and associated with poor survival and advanced stages (Jun et al. 2009). Heterodimerization between IGF-1R and EGFR occurs in SCCHN cells when stimulated by either insulin-like growth factor (IGF) or EGF (Barnes et al. 2007). Furthermore combined blockade of both the IGF-1R and the EGFR was more effective than blocking each one individually in a SCCHN xenograft mouse tumor models (Barnes et al. 2007). **Cixutumumab**, a human IgG1 monoclonal antibody targeting the IGF1-R was investigated in a phase II trial with recurrent and/or metastatic SCCHN (Glisson et al. 2013). Patients were randomized to cixutumumab alone or to cixutumumab and cetuximab. No significant activity was found, confirming the absence of activity of IGF-1R inhibitors in unselected patients with recurrent SCCHN (Schmitz et al. 2012).

6.4.2 The c-Met Pathway

c-Met is a transmembrane tyrosine receptor primarily expressed on epithelial cells that dimerizes and autophosphorylates after activation by the hepatocyte growth factor (HGF) secreted by mesenchymal cells. This epithelial-mesenchymal interaction leads to the activation of the receptor, which mediates downstream signaling through phosphoinositide 3-kinase, phospholipase C-γ1, STAT3, focal adhesion kinase, tyrosine phosphatase SHIP-2, mitogen-activated protein kinase, p38, c-Jun N-terminal kinases (JNK), and nuclear factor κB (Trusolino et al. 2010). Around 75% of SCCHN overexpress c-Met (Chau et al. 2011; Seiwert et al. 2009) and overexpression was associated with low response to EGFR inhibitors in SCCHN

and decreased survival rates (Chau et al. 2011; Kim et al. 2010). Activating *Met* mutations have been identified in about 14% of primary SCCHN (Seiwert et al. 2009; Ghadjar et al. 2009).

Furthermore c-Met is implicated in resistance to EGFR inhibitors through maintenance of activation of the downstream signaling pathways of EGFR by amplification of the *Met* oncogene (Engelman et al. 2007; Turke et al. 2010), and/or increased activation and expression of c-Met (Benedettini et al. 2010; Agarwal et al. 2009). It has been also reported that overexpression of cortactin, a key regulator of dynamic actin networks, stabilized c-Met in SCCHN cell lines, enhanced HGF-induced mitogenesis and cell scattering, and led to gefitinib resistance (Timpson et al. 2007). In addition, amplification of c-Met may cause gefitinib resistance by driving ERBB3 (HER3)-dependent activation of PI3K in lung cancer (Wheeler et al. 2008). Xu et al. found that the combination of c-Met inhibitor PF2341066 with the EGFR inhibitor gefitinib abrogated in vitro SCCHN cell proliferation, invasion, and wound healing significantly more than with the inhibition of each pathway alone. The investigators also showed TGF-α induced phosphorylation of c-Met in the absence of HGF, supporting a ligand-independent mechanism (Xu et al. 2011).

6.4.3 Inhibitors of Angiogenesis

Angiogenesis is an important process for primary tumor growth, cell proliferation and metastatic spread. Under hypoxic conditions multiple growth factors, including the VEGF, platelet derived growth factor (PDGF), fibroblast growth factor beta (FGF)-β, transforming growth factor beta (TGF)-β, placental growth factor (PIGF), and angiopoietin (AP)-2 (Calvani et al. 2006; Lewis and Hughes 2007; Jiang et al. 2007), are released by cancer cells.

Sorafenib is a multikinase inhibitor of VEGFR, PDGFR, Raf and c-kit kinase. A phase II trial comparing cetuximab alone to the combination of cetuximab and sorafenib is ongoing in patients with refractory, R/M SCCHN (NCT00939627).

Vandetanib is a tyrosine kinase inhibitor targeting VEGFR-2, EGFR and rearranged during transfection (RET). Sano et al. (2007, 2011) demonstrated that vandetanib displayed antiproliferative effects on SCCHN cells and induced apoptosis and antiangiogenic activity on nude mice bearing an established YCU-H891 xenograft. They also demonstrated that vandetanib plus cisplatin radiosensitized SCCHN cells in vitro and in vivo. The combination of vandetanib, cisplatin, with radiation was superior to either each of this treatment as monotherapy or a combination of 2 of these treatments in terms of antitumoral effects, prolonged survival and regional metastases in vivo. Studies combining vandetanib, radiotherapy and cisplatin in advanced SCCHN are completed (NCT00720083, NCT00450138) but final results are not yet available.

6.4.4 Inhibitors of the PI3K/Akt/mTOR Pathway

Tumor arrays analysis (Molinolo et al. 2007) showed activation of Akt-mammalian target of rapamycin (mTOR) pathway in 90% of SCCHN. A small subgroup of tumors have activated mTOR pathway without Akt activation, suggesting the existence of an Akt-independent stimulator of mTOR. Gupta et al. (2002) found a significant association between low p-Akt staining and local control in 38 patients treated with radiotherapy ($p = 0.04$).

Concerning PI3K inhibitors, one study explored BYL719 (NCT01602315) plus cetuximab. Preliminary results were presented at ASCO 2014 and showed that combined inhibition was well tolerated with encouraging antitumor activity (Razak et al. 2014). Patients were treated either by tablets (ARM A) or suspension (ARM B) of BYL719, 300 mg once daily The overall DCR was 60% (12/20 pts) and 47.1% (8/17), respectively.

A mTOR inhibitor, **rapamycin**, showed antiproliferative effects and induced apoptosis in SCCHN cell lines (Cassell et al. 2012). Other mTOR inhibitors displayed synergistic and additive effects in preclinical models when combined with EGFR inhibitors (Cassell et al. 2012), bevacizumab, cetuximab or radiotherapy (Bozec et al. 2011), or when used in combination with paclitaxel or carboplatin (Aissat et al. 2008).

In a phase II study, Bauman et al. evaluated the combination of oral erlotinib (150 mg daily), and **temsirolimus** (15 mg, intravenously, weekly), a mTOR inhibitor, in patients with platinum-refractory recurrent and/or metastatic SCCHN. The study was closed early after enrolling 12 patients due to high toxicity. Grade ≥ 3 toxicities included fatigue (n = 5), diarrhea (n = 2), gastrointestinal infection/peritonitis (n = 2), dyspnea (n = 2), head and neck edema (n = 2), and neutropenia (n = 1). Among 8 evaluable patients, median PFS was 1.9 months. Median OS was 4.1 months (Bauman et al. 2012).

6.4.5 Inhibitors of the Signal Transducer and Activation of Transcription (STAT) Pathway

The STAT pathway is another interesting pathway that can be activated by Src, a nonreceptor cytoplasmic tyrosine kinase that is activated by signals from cell surface receptors, such as EGFR. Different preclinical studies suggested that STAT-3 may be implicated in resistance to radiation and cetuximab (Bonner et al. 2011; Sen et al. 2012). **Dasatinib**, an inhibitor of c-src, was investigated in vitro. Results showed induction of apoptosis, blockage of DNA repair in EGFR-expressing SCCHN cells, and sensitization of SCCHN cell lines to radiation (Raju et al. 2012). However, single administration of dasatinib failed to demonstrate any clinical activity in patients with advanced SCCHN. Currently a preoperative window study comparing single agent erlotinib or dasatinib to placebo to the combination of both

drugs is ongoing (NCT00779389). First results, presented at ASCO 2014 showed that high pMAPK expression was associated with reduced tumor size for patients treated with erlotinib without an independent or added effect of dasatinib (Bauman et al. 2014). Another study with a Src kinase inhibitor **Saracatinib**, (175 mg daily) had to be stopped earlier than planned due to lack of efficacy (Fury et al. 2011). A preclinical study showed that increased EGFR activation by ligand administration rescued cells from dasatinib-induced apoptosis, whereas inhibition of EGFR enhanced its apoptotic effect (Lin et al. 2012). This could be one possible explication for the poor clinical activity seen with Src inhibitors as monotherapy in SCCHN.

6.4.6 Inhibitors of Heat Shock Proteins

Heat shock proteins (HSP) are a class of functionally related proteins involved in the folding and unfolding of other proteins, including oncoproteins. Their expression is increased when cells are exposed to stress. NVP-AUY922, an HSP90 inhibitor, was shown to inhibit proliferation of oral squamous cell carcinoma cell lines. Antitumor activity was mediated by the degradation of client proteins inducing ErbB2, p-Akt, p-S6, HIF1-α and VEGF and increased expression of cleaved caspase-3 and apoptosis (Okui et al. 2011). Suppression of p-Akt and VEGF expression in a xenograft model was also documented after administration of the HSP90 inhibitor (Okui et al. 2011). Another group showed radiosensitization in a human SCCHN xenograft model treated by the same HSP 90 inhibitor (Zaidi et al. 2012). Interaction of HSP90 with EGFR was found to be critical for maintaining receptor stability as well as growth of EGFR-dependent cancer (Ahsan et al. 2012). These findings support the potential role of HSP90 inhibitors for the treatment of SCCHN. An ongoing study is investigating the combination of cetuximab and IPI-926, an HSP inhibitor, in patients with R/M SCCHN (NCT01255800).

6.5 Conclusion

The anti-EGFR mAb Cetuximab has been shown to improve overall survival either as curative treatment in combination with radiation therapy or as palliative treatment in combination with chemotherapy. Although some patients might benefit from anti-EGFR monoclonal antibodies in R/M SCCHN, the majority of tumors will have already developed primary resistance, and ultimately all will develop acquired resistance. Currently other anti-EGFR agents with potential advantages over Cetuximab are under investigation in various treatment settings. A new approach to investigate these new compounds is to design so called window of opportunity studies in order to detect early activity in treatment naïve patients and to determine pharmacodynamic modifications. All these studies are conducted with

the objective to better understand molecular mechanisms involved in treatment resistance to anti-EGFRs and to identify predictive parameters of response.

The use of combination or sequential targeted therapies targeting not only the cell but also compounds of the microenvironment may ultimately be necessary to abrogate treatment resistance.

Furthermore upfront stratification of study patients based on specific genetic or molecular alteration would also increase the efficiency of such trials.

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Chapter 7
Targeting the mTOR Signaling Circuitry in Head and Neck Cancer

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Abstract The recent development of deep-sequencing approaches for the study of human cancer represents a revolution in medical oncology. These approaches provide an unprecedented knowledge of the multiplicity of genomic and epigenetic alterations underlying each cancer type, and enable the elucidation of aberrant molecular networks driving tumor progression. The in-depth analysis of the head and neck cancer (HNSCC) oncogenome revealed that most HPV negative and HPV positive HNSCC lesions exhibit mutations and copy number alterations converging in the aberrant activation of the PI3K-mTOR pathway. In turn, this overreliance on PI3K-mTOR signaling for tumor growth can expose a cancer vulnerability that can be exploited for therapeutic purposes. Indeed, we have shown that the vast majority of HNSCC lesions exhibit highly active PI3K-mTOR pathway. Furthermore, we have shown that the administration of mTOR inhibitors causes tumor regression and prevents the malignant conversion of oral precancer lesions in multiple chemically induced and genetically defined HNSCC animal models. Many PI3K-mTOR pathway inhibitors have been recently advanced to the clinic with

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_7

encouraging results in HNSCC. Overall, we can expect that the use of PI3K-mTOR inhibitors will provide an opportunity for precision medicine approaches for HNSCC prevention and treatment.

7.1 Introduction

The elucidation of the dysregulated molecular mechanisms driving HNSCC progression may afford a unique opportunity to identify novel targeted treatment options for HNSCC therapy and prevention. Several molecular targets have been tested in clinical trials and this chapter presents novel research on the efficacy of mTOR inhibition in cancer treatment and prevention. Other approaches that are emerging to targeting mTOR, for example with metformin also requires careful consideration.

7.2 Activation of the EGFR-PI3K-AKT-mTOR Signaling Network in Head and Neck Cancer

EGFR—presented in Chap. 6—regulates multiple intracellular signaling circuits, including the JAK/STAT, RAS/MAPK, and PI3K/AKT/mTOR pathways (Sharma et al. 2007; Tebbutt et al. 2013; Maulik et al. 2002). Among them, we have provided evidence that the persistent activation of PI3K/AKT/mTOR signaling circuitry is one of the most frequent dysregulated molecular events HNSCC lesions (Amornphimoltham et al. 2005; Molinolo et al. 2007a; Molinolo et al. 2012; Iglesias-Bartolome et al. 2013) (Fig. 7.1). This pathway involves the sequential activation of Phosphoinositide 3-kinase (PI3K), AKT, and mTOR. PI3Ks are grouped into three classes (I–III) according to their substrate preference and sequence homology (Cantley 2002). The class I PI3Ks are activated by growth factor tyrosine kinase receptors (class IA), such as EGFR, or by G protein coupled receptors (GPCRs) (class IB). Class IA PI3Ks are heterodimers of a p85 regulatory subunit and a p110 catalytic subunit. The direct product of PI3K activity is the lipid second messenger PtdIns (3,4,5)P_3 (PIP_3), which serves as docking sites for proteins that contain PH domains, including AKT and phosphoinositide-dependent kinase 1 (PDK1) (Cantley 2002; Sarbassov et al. 2006) which phosphorylates AKT in threonine 308 ($pAKT^{T308}$) resulting in its activation. A second activation-specific AKT phosphorylation in serine 473 ($pAKT^{S473}$) is targeted by mTOR as part of its complex 2 (mTORC2) (Sarbassov et al. 2006). In turn AKT plays a key role in the transmission of pro-proliferative and transforming pathways initiated by EGFR and multiple growth factor receptors, as well as by oncogenic active PI3K mutants (Luo et al. 2003).

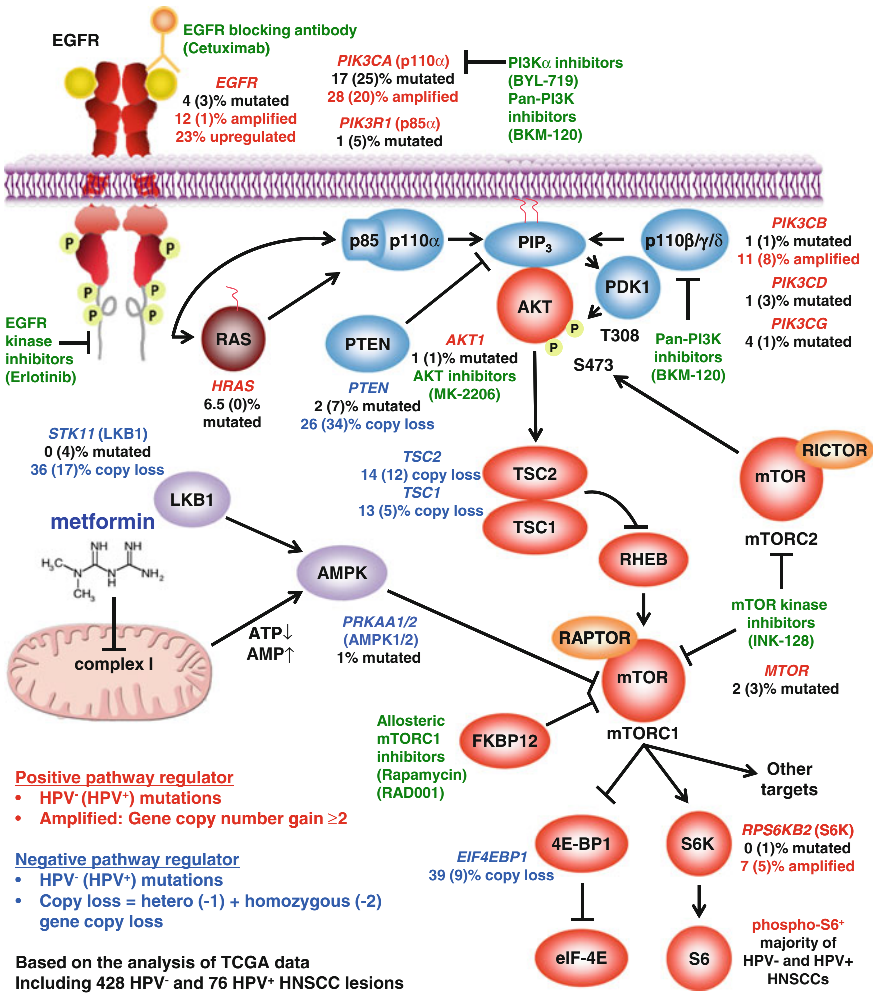

Fig. 7.1 Genetics alterations in EGFR/PI3 K/mTOR signaling circuitry in HPV^- and HPV^+ HNSCC lesion—precision therapeutic targets for HNSCC treatment

AKT prevents cell death by inactivating proapoptotic factors including BAD, procaspase-9 and Forkhead transcription factor family proteins (FOXOs), activates transcription factors that upregulate antiapoptotic genes, including NF-κB, inactivates p53 through Mdm2, and phosphorylates the cell cycle inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, thus increasing cell proliferation (Hennessy et al. 2005). AKT also phosphorylates and inhibits glycogen synthase kinase-3 (GSK3), thus enhancing β-catenin and cyclin D1 stabilization (Vivanco and Sawyers 2002). In this regard, we provided early evidence that AKT is persistently activated in the vast majority of HNSCC cases. Specifically, we showed that both experimental and human

HNSCCs and HNSCC-derived cell lines exhibit a remarkable increase in the levels of phosphorylated AKT (Amornphimoltham et al. 2004), and that blockade of PDK1, which acts upstream of AKT, inhibits tumor cell growth (Amornphimoltham et al. 2004; Patel et al. 2002). AKT activation was subsequently found to represent an early event in HNSCC progression. Active AKT can be detected in 50% of tongue preneoplastic lesions (Massarelli et al. 2005), and AKT activation can be considered an independent prognostic marker of poor clinical outcome in tongue and oropharyngeal HNSCC (Massarelli et al. 2005; Yu et al. 2007).

While AKT phosphorylates multiple downstream targets (see above), the emerging picture is that the ability of AKT to coordinate mitogenic and nutrient-sensing pathways controlling protein synthesis is a key mechanism by which AKT regulates cell proliferation. As depicted in Fig. 7.1, AKT phosphorylates and inactivates the tumor-suppressor protein tuberous sclerosis complex protein 2 (TSC2), which forms a complex with tuberous sclerosis complex protein 1 (TSC1), and act together as a GTPase activating protein (GAP) for the small GTPase Rheb1 (Inoki et al. 2003, 2005a, b). AKT phosphorylation and inactivation of TSC2 results in increased levels of the GTP-bound (active) form of Rheb1, which in turn promotes the phosphorylation and activation of mTOR, also known as the mammalian target of rapamycin (Manning and Cantley 2003). Subsequently, mTOR phosphorylates key eukaryotic translation regulators, including p70-S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Hay and Sonenberg 2004). The latter overrides the repressing activity of 4E-BP1 on the eukaryotic initiation factor 4E (eIF4E), resulting in enhanced translation of a subset of growth promoting genes (Hay and Sonenberg 2004). This is of particular relevance to HNSCC, as eIF4E gene amplification and protein overexpression is often associated with malignant progression (Sorrells et al. 1999), and eIF4E-positive surgical margins have increased risk of developing local recurrences (Nathan et al. 2002, 2004). Furthermore, supporting the relevance of the PI3K-AKT-mTOR pathway in HNSCC, we showed that the accumulation of the phosphorylated form of the ribosomal S6 protein, pS6, a typical downstream target of the mTOR pathway, is an early and one of the most frequent events in HNSCC (Molinolo et al. 2007b; Amornphimoltham et al. 2005). As part of an International HNSCC Tissue Array initiative, including HNSCC lesions from eight countries including Argentina, China, Japan, India, Mexico, South Africa, Thailand, and United States, we also observed that mTOR activation is a widespread event in HNSCC, irrespective of the risk factors associated with HNSCC initiation and progression in each country or geographical region (Molinolo Molinolo et al. 2007a, b). As described below, we also observed that inhibition of mTOR with specific inhibitors, such as rapamycin, provokes the rapid regression of multiple HNSCC xenografts (Amornphimoltham et al. 2005). These early findings provided a strong rationale for the preclinical and clinical evaluation of the efficacy of mTOR inhibiting strategies for HNSCC prevention and treatment.

7.3 mTOR Activation in HPV-Associated HNSCC

Human papillomavirus (HPV) infection is now recognized as a key etiologic factor for HNSCC (D'Souza et al. 2007; Gillison and Shah 2001). This is of particular importance in many areas of the world, including United States, as the overall incidence of HNSCC has decreased in parallel with a decrease in tobacco use, but an alarming increase of HNSCC arising in the oropharynx, the most frequent HPV-associated anatomical site, has been observed, particularly among young patients (Chaturvedi et al. 2008; Ryerson et al. 2008; Shiboski et al. 2005). HPV-associated HNSCC, with HPV16 representing the primary HPV type involved, frequently arise in the oropharynx, and represent a clinical and anatomically distinct oral cancer type (Gillison et al. 2000).

The HPV^+ subset of HNSCC lesions are initiated by the HPV oncoproteins E6 and E7, which inhibit TP53 and RB tumor suppressor proteins, respectively (Werness et al. 1990; Huang et al. 1993). However, E6 and E7 cannot be therapeutically targeted, making it critical to identify alternative pathways that are relevant and druggable in HPV-related malignancies. We found that most HPV^+ HNSCC exhibit elevated mTOR activity, as judged by the accumulation of pS6 and inactive p4EBP1 (Molinolo et al. 2012). As described below, these observations prompted us to investigate whether mTOR inhibitors (rapamycin and RAD001) represent suitable therapeutic options for HPV^+ HNSCC, leading to the discovery that mTOR inhibition can have remarkable growth inhibitory activity in preclinical HPV^+ HNSCC models (Molinolo et al. 2012).

7.4 The Emerging Genomic Landscape of Head and Neck Cancer

The recent development of deep-sequencing approaches for the study of human cancer genomes represents a revolution in medical oncology (Garraway and Lander 2013). These approaches provide an unprecedented knowledge of the multiplicity of genomic and epigenetic alterations underlying each individual cancer lesion, and enable the elucidation of aberrant molecular mechanisms and signaling circuitries driving tumor progression. This remarkable body of information has already revealed novel druggable targets for therapeutic interventions in many human malignancies. The evidence emerging from recent reports (Pickering et al. 2013; Lui et al. 2013b; Agrawal et al. 2011; Stransky et al. 2011) and the landmark study from the HNSCC Cancer Genome Anatomy (TCGA) effort (The Cancer Genome Atlas 2015), has also revealed the multiplicity and diversity of genetic alterations in HNSCC. This makes the search for cancer-driving molecular events daunting, especially regarding the ability to distinguish them from passenger mutations that may have minimal impact on tumor progression and/or clinical response. However, the in-depth analysis of the HNSCC oncogenome suggest that in spite of the

complexity of the distinct molecules alterations in individual lesions, they all participate in a limited number of molecular pathways whose dysregulation may contribute to most HNSCC cases.

Specifically, most of alterations identified in HPV^{-} HNSCC fall within four major driver biologic processes: (i) mitogenic signaling (63%), with particular emphasis on aberrant activation of the PI3K/mTOR pathway (including 17% with mutations of *PIK3CA*, encoding the catalytic subunit of PI3Kα); (ii) defective cell differentiation (including 9% with *NOTCH1* gene mutations and 66% with predicted NOTCH signaling pathway alterations); (iii) nearly universal (94%) cell-cycle deregulation due to inactivation of the *CDKN2A* (*p16*$^{\text{INK4A}}$) tumor suppressor gene by copy number loss or promoter methylation, together with *CCND1* (CYCLIN D1) amplification; and (iv) genomic instability caused by loss of *TP53* and other candidate genes, such as those involved in DNA damage recognition and repair. In addition, HNSCC harbor mutations in genes likely affecting cell–cell communication and cell death: *FAT1* (30%) and *CASP8* (10%), respectively. This reductionist approach to catalog the wealth of sequencing information may facilitate the identification of actionable targets of relevance to large subgroups of HNSCC patients whose alterations may converge in key druggable molecular processes.

7.5 Multiple Genomic Alterations in Head and Neck Cancer Converge in the PI3K-mTOR Pathway

If one analyses the genomic alterations in HNSCC in a pathway-specific fashion, most HNSCC lesions exhibit mutations and copy number alterations converging in the aberrant activation of the PI3K-mTOR pathway (Fig. 7.1). In contrast, the MAPK and JAK/STAT pathways are mutated in less than 10% of the cases (Lui et al. 2013b). *PIK3CA* is the most mutated gene in the pathway when considering HPV^{-} and HPV^{+} HNSCC lesions (16.8%, Table 7.1), and mutations in PI3K genes (*PIK3CA, PIK3CB, PIK3CD and PIK3CG*) are the most frequent mutated oncogenes in 30% of the HPV^{+} tumors. However, as shown in Fig. 7.1 and Table 7.1, *PIK3CA* mutations are the most frequent but not the only genomic alterations resulting in the persistent activation of the PI3K-AKT-mTOR pathway in HNSCC. Indeed, multiple genetic and epigenetic changes act in concert with *PIK3CA* mutations to sustain pathway activation in HNSCC (Fig. 7.1).

For example, *PIK3CA* gene copy number gain (within 3q) and mRNA overexpression are frequent events in HNSCC (20 and 52%, respectively). Mutations and copy number gains can also occur in other PI3K isoforms and in multiple PI3K regulatory subunits (0.5–11%), and few HNSCC lesions have mutations in AKT, mTOR and its associated subunits, RICTOR and RAPTOR, and the tumor suppressor genes *TSC1* and *TSC2*. In addition, mutations and gene copy number loss are also observed in the tumor suppressor *PTEN* in HNSCC (2 and 26%,

Table 7.1 Gene alterations in the EGFR, PI3 K, AKT, mTOR signaling circuitry in HPV^- and HPV^+ HNSCC lesions

Genes	HPV^- (n = 428)		HPV^+ (n = 76)	
	Mutations (%)	CNV (gain) (%)	Mutations (%)	CNV (gain) (%)
EGFR	4.2	12.1	2.6	1.3
HRAS	6.5	0.7	0.0	0.0
KRAS	0.2	1.6	0.0	0.0
NRAS	0.2	0.0	0.0	0.0
MTOR	2.1	0.5	2.6	0.0
PDPK1	0.9	0.5	0.0	0.0
AKT1	0.2	1.6	1.3	0.0
AKT2	0.9	1.2	0.0	1.3
AKT3	1.2	1.9	0.0	1.3
PIK3CA	16.8	20.1	25.0	27.6
PIK3CB	1.4	11.0	1.3	7.9
PIK3CG	3.7	3.3	1.3	0.0
PIK3CD	1.4	0.5	2.6	0.0
PIK3R1	1.2	0.0	5.3	0.0
PIK3R2	0.9	0.5	0.0	0.0
PIK3R3	0.9	0.2	1.3	0.0
RHEB	0.2	0.5	0.0	0.0
RPS6KB1	0.9	0.0	1.3	1.3
RPS6KB2	0.2	6.5	1.3	5.3
RICTOR	1.6	6.3	2.6	1.3
RAPTOR	1.4	0.2	1.3	1.3
EIF4E	0.2	0.0	0.0	0.0
PIK3R4	2.3	5.8	0.0	5.3
PIK3R5	1.6	0.0	2.6	0.0
PIK3R6	0.5	23.1	1.3	0.0

Genes	HPV^- (n = 428)			HPV^+ (n = 76)		
	Mutations (%)	CNV het (loss) (%)	CNV homo (loss) (%)	Mutations (%)	CNV het (loss) (%)	CNV homo (loss) (%)
PTEN	2.1	26.2	1.6	6.6	34.2	14.5
STK11	0.5	35.7	1.2	4.0	17.1	1.3
TSC1	0.7	13.3	0.0	1.3	5.3	0.0
TSC2	1.6	13.8	0.0	1.3	11.8	0.0
EIF4EBP1	0.2	39.5	1.6	0.0	9.2	0.0

Genes in the EGFR, PI3K, AKT, mTOR pathway were analyzed for mutations and copy number variations (CNV) depending from their HPV status. In the upper table, genes that have a positive impact on the pathway were analyzed for gene copy number gain, while in the lower table, genes repressing the pathway were analyzed for copy number loss, depicting a single gene copy (heterozygous, Het) or homozygous (Homo) gene loss. Data were computed from HNSCC TCGA

respectively), which likely result in PI3K pathway activation (Lui et al. 2013a). We have also documented that PTEN protein expression is reduced in approximately 30% of HNSCCs (Molinolo et al. 2012; Squarize et al. 2013), suggesting that PTEN functional inactivation is a shared feature of a subset of HNSCCs. Overall, the PI3K-mTOR is a major HNSCC driver. In turn, this overreliance on PI3K-mTOR signaling for tumor growth can expose a cancer vulnerability that can be exploited for therapeutic purposes. As depicted in Fig. 7.1, multiple inhibitors of the pathway have been already developed, many of which have been already advanced to the clinic with encouraging results (Table 7.2 and see below).

7.6 Effectiveness of mTOR Inhibitors in Experimental HNSCC Models

Our group was among the first to establish that mTOR inhibition, using rapamycin as a single agent, is effective for treating HNSCC using human HNSCC tumor xenografts in vivo. We showed that inhibition of mTOR by rapamycin causes the rapid decrease in the level of pS6 and the apoptotic death of HNSCC tumor xenografts, thereby causing rapid tumor regression (Amornphimoltham et al. 2005). Since our initial HNSCC xenograft study, multiple groups have reported the anti-cancer effect of rapalogs alone or in combination with chemotherapy or radio-therapy in HNSCC xenografts (Ekshyyan et al. 2009; Shin et al. 2011; Bonner et al. 2006; Cassell et al. 2012; Zhong et al. 2014; D'Amato et al. 2014) including HNSCC models of minimal residual disease (MRD) (Nathan et al. 2007). We then challenged our prior observations in more complex chemically induced and genetically defined mouse SCC experimental models. Initially we used the well-established DMBA-TPA two-step chemical carcinogenesis model in which chronic administration of rapamycin remarkably decreased the tumor burden and prolonged the survival of mice harboring early and advanced primary tumor lesions, and even recurrent SCC (Amornphimoltham et al. 2008). Immunohistochemical studies on tumor biopsies and clustering analysis enabled investigating the relationship among molecular changes caused by mTOR inhibition, thus helping to identify relevant biomarkers for monitoring the effectiveness of mTOR inhibition in the clinical setting. In a follow up study, we developed and optimized a novel oral-specific chemical carcinogenesis model in mice that can recapitulate human HNSCC by using 4-Nitroquinoline-1 oxide (4NQO) in the drinking water. 4NQO which causes DNA lesions resembling tobacco carcinogens and hence is considered a tobacco mimetic (Czerninski et al. 2009). Mice develop exclusively oral tumors, mostly in the tongue, and many of these lesions evolve spontaneously into highly malignant SCCs within a few weeks after 4NQO withdrawal. This approach provided a facile oral-specific chemical carcinogenesis model system for the evaluation of molecular targeted approaches for oral cancer prevention and treatment. In this model, we showed that the activation of the Akt-mTOR pathway represents an early

Table 7.2 HNSCCclinical trials targeting in the PI3K/mTOR pathway

Clinical trial ID	Drugs	Combination	Phase	Status	Conditions
NCT00195299	Temsirolimus	Single agent	0	Complete	Newly diagnosed advance HNSCC
NCT00703625	Temsirolimus	Docetaxel	I	Complete	Resistant solid malignancies
NCT00858663	Everolimus	RT + cisplatin	I	Complete	Advanced HNSCC
NCT00935961	Everolimus	Docetaxel + cisplatin	I	Complete	Local-regional advanced HNSCC
NCT00942734	Everolimus	Erlotinib	II	Completed	Recurrent HNSCC
NCT01009203	Temsirolimus	Erlotinib	II	Terminated	Platinum-refractory, advanced HNSCC
NCT01009346	Everolimus	Cetuximab + cisplatin	I/II	Terminated	Recurrent or metastatic HNSCC
NCT01015664	Temsirolimus	Cetuximab + cisplatin	I/II	Terminated	Recurrent or metastatic HNSCC
NCT01016769	Temsirolimus	Carboplatin + paclitaxel	I/II	Ongoing	HNSCC
NCT01016769	Temsirolimus	Paclitaxel + carboplatin	I/II	Ongoing	Recurrent or metastatic HNSCC
NCT01051791	Everolimus	Single agent	II	Ongoing	HNSCC
NCT01057277	Everolimus	RT + cisplatin	I	Terminated	Locally advanced HNSCC
NCT01058408	Everolimus	RT + cisplatin	I	Terminated	Locally advanced HNSCC
NCT01133678	Everolimus	Single agent	II	Ongoing	HNSCC
NCT01172769	Temsirolimus	Single agent	II	Completed	HNSCC
NCT01195922	Rapamycin	Single agent	I/II	Completed	Previously untreated HNSCC
NCT01212627	Ridaforolimus	Cetuximab	I	Terminated	Advanced HNSCC, non-small cell lung cancer and colon cancer
NCT01256385	Temsirolimus	Cetuximab	II	Completed	Recurrent or metastatic HNSCC
NCT01283334	Everolimus	Carboplatin + cetuximab	I/II	Completed	Advanced HNSCC
NCT01313390	Everolimus	Docetaxel	I/II	Terminated	Recurrent, locally advanced or metastatic HNSCC
NCT01332279	Everolimus	RT + Erlotinib	I	Terminated	Recurrent HNSCC with previously RT
NCT01333085	Everolimus	Carboplatin + paclitaxel	I/II	Completed	Unresectable or inoperable locally advanced HNSCC
NCT01333852	Metformin	Placlitaxel	II	Terminated	Recurrent or metastatic HNSCC

(continued)

Table 7.2 (continued)

Clinical trial ID	Drugs	Combination	Phase	Status	Conditions
NCT01341834	RAD001	LBH589	I	Active	EBV^{+} solid tumors
NCT01349933	MK2206	Single agent	II	Completed	Recurrent/stage IV SCC of the nasopharynx
NCT01353625	CC-115	Single agent	I	Active	Advanced solid tumors
NCT01602315	BYL719	Cetuximab	I/II	Active	Platinum-refractory, recurrent or metastatic HNSCC
NCT01637194	Everolimus	Cetuximab	I	Completed	Metastatic or recurrent HNSCC or colon cancer
NCT02083692	Metformin	Single agent	0	Recruiting	HNSCC
NCT02145312	BYL719	Single agent	II	Not yet recruiting	Platinum-refractory, recurrent or metastatic HNSCC
NCT02215720	Temsirolimus	Cetuximab	I	Recruiting	Advanced or metastatic solid tumors
NCT02325401	Metformin	Cisplatin + radiation	I	Recruiting	Locally advanced HNSCC
NCT02402348	Metformin	Single agent	0	Recruiting	HNSCC
NCT02439489	BKM120	Cisplatin or carboplatin	I	Recruiting	Advanced solid tumors
NCT02537223	BYL719	Cisplatin + radiation	I	Active	Locoregionally advanced HNSCC
NCT02581137	Metformin	Single agent	2	Not yet recruiting	Erythroplakia, oral leukoplakia, oral SCC

Current list of clinical trials targeting the PI3K/mTOR pathway in HNSCC and potential oral premalignant lesions, as single agents or as part of combination therapies. *RT* radiation therapy; *HNSCC* head and neck squamous cell carcinoma; *SCC* squamous cell carcinoma; *EBV* Epstein-Barr virus

event already detectable in dysplastic lesions. Inhibition of mTOR by rapamycin administration halted the malignant conversion of precancerous lesions and promoted the regression of advanced carcinogen-induced SCCs.

These findings prompted us to direct our efforts towards the development of genetically defined animal models in which we could conditionally activate the Akt-mTOR signaling route. Indeed, when Pten, a PIP_3 lipid phosphatase, was conditioned deleted from the epithelial compartment (Squarize et al. 2013), it was sufficient to cause multiple hyperproliferative and tumor lesions in the skin and mouth and resulted in early animal lethality. Remarkably, the prolonged administration of rapamycin in low doses caused the rapid regression of advanced tumor lesions, and was sufficient to prevent tumor development if initiated before disease manifestation. These findings lent support to a recently opened clinical trial using rapamycin in Cowden's disease patients, who harbor inactivating mutations in *PTEN* and hence develop multiple benign tumors and are predisposed to cancer. In parallel, after a systematic analysis of available tamoxifen-inducible Cre recombinase systems (CreERTM) we developed an oral carcinogenesis model in which this gene is expressed under the control of the K14 promoter (K14-CreERTM) and mice in which the *ras* oncogene is expressed from its own promoter after Cre excision of a transcription stop signal. These mice develop large tumors, primarily benign papillomas, exclusively in the oral cavity within 1 month of a brief tamoxifen treatment, and when combined with a *tp53* conditional knockout background, the compound mice develop carcinomas exclusively on the tongue as early as 2 weeks after tamoxifen induction (Raimondi et al. 2009). We showed that the activation of mTOR signaling pathway is an early event in both oral benign and malignant lesions and that targeting mTOR by the use of rapamycin halts tumor progression in this genetically defined oral cancer model, thereby prolonging animal survival.

In our efforts to address the effects of mTOR inhibition with rapamycin in advanced HNSCC, an orthotopic tumor model that spontaneously metastasize to the cervical lymph nodes was developed, where the presence of metastatic HNSCC cells can be detected by histological evaluation. We found that inhibition of mTOR with rapamycin diminishes lymphangiogenesis in the primary tumors and prevents the dissemination of HNSCC cancer cells to the cervical lymph nodes, thus prolonging animal survival (Patel et al. 2011).

More recent studies have extended the repertoire of HNSCC and SCC animal model systems in which mTOR inhibition was found to be effective to halt cancer progression. This includes the analysis of the interplay between the TGF-β and Akt-mTOR pathway in which we conditionally deleted *Tgfbr1* and *Pten* in mouse oral mucosa using the Cre-LoxP approach (K14-CreERTM) resulted in HNSCC development with mTOR complex 1 activation. We showed that rapamycin treatment delayed tumorigenesis by inhibiting activation of mTOR and survivin

expression, resulting in prolonged mice survival. (Sun et al. 2012.). We have also identified the role of c-Met and mTOR in matriptase-induced SCC in which matriptase activates c-Met by increasing the conversion of proHGF to HGF, the active c-Met ligand. The molecular dissection revealed mTOR activation as an essential component of matriptase/c-Met-induced carcinogenesis and rapamycin treatment completely prevented matriptase-mediated tumorigenesis (Szabo et al. 2011).

Patient derived tumorgrafts (PDX) model have been recently recognized to better predict clinical outcome since it can maintain comparable gene expression patterns and retain tumor heterogeneity profile of a primary tumor from which they are derived. In a recent large comprehensively characterized HNSCC PDX model study, everolimus, a rapamycin-related mTOR inhibitor (rapalog) showed a significant growth inhibition in 20 of 29 (68%) PDX models when used as single agents (Klinghammer et al. 2015).

7.7 Inhibiting mTOR for HNSCC Treatment in the Clinic

Overall, our recent findings and published results from many laboratories have provided a strong rationale for the use of mTOR inhibitors as a molecular-targeted approach for the treatment of HNSCC. Ongoing clinical trials investigating rapalogs as single agents or the combination of everolimus or temsirolimus with radiation, chemotherapeutic drugs or other inhibitors in HNSCC are summarized in Table 7.2. While most of the studies are yet to be completed or reported, our clinical study using rapamycin (sirolimus) in newly diagnosed HNSCC patients (NCT01195922; Table 7.2) has already provided encouraging results. Patients with untreated stage II-IVA HNSCC received 21 days of rapamycin treatment prior to definitive treatment with surgery or chemoradiation. Sixteen patients (8 oral cavity, 8 oropharyngeal) completed rapamycin and definitive treatment (15-surgery, 1-chemoradiation). Half of patients were p16 positive, likely reflecting HPV^{+} HNSCC cases. Fifteen of 16 patients showed clinical improvement. One patient had pathological complete response and three met RECIST criteria for response (1 CR, 2 PR, 14 SD). Treatment was well tolerated with no grade 4 or unexpected toxicities. Our studies revealed that mTOR inhibition with rapamycin treatment was well tolerated and resulted in significant clinical responses despite the brief treatment duration. Significant reduction in mTOR activation was observed, supporting the emerging notion that mTOR may represent a druggable target for HNSCC treatment, either as using mTOR inhibitors as single agents or as part of co-targeting strategies.

7.8 Targeting mTOR with Metformin for Oral Cancer Prevention

In spite of the strong rationale for the use of PI3K and mTOR inhibitors for HNSCC in the clinic, their potential immunosuppressive activity and other undesirable side effects may raise safety concerns regarding their long term use as chemopreventive agents (Cohen et al. 2012). This is of particular relevance to patients diagnosed with potential premalignant oral lesions (OPL), such as hyperplasia and dysplasia that may undergo variable progression to malignancy over a period of years, thus requiring extended therapy (Warnakulasuriya et al. 2008). OPL often present as leukoplakia or erythroplakia (white or red patches, respectively) that have variable rates of progression to cancer, ranging from 11 to 36% for leukoplakia to >50% for erythroplakia (Lee et al. 2000; Reichart and Philipsen 2005). Long-term surveillance for progression is frequently the choice for non-resectable OPL patient management. Furthermore, even adequate surgical resection with negative margins has a relatively high rate (15–40%) of progression to oral squamous cell carcinoma (Arnaoutakis et al. 2013). This reinforces the concept that the presence of OPL represents a risk for malignant transformation due to occult clonal premalignant cells that may demonstrate normal histology (Braakhuis et al. 2005).

Metformin is an oral biguanide that is currently the drug of choice for the treatment of Type 2 Diabetes, and is being prescribed to at least 120 million people worldwide (Viollet et al. 2012). Hence, metformin's safety profile for long-term use and the management of its potential side effects are all well documented. Compelling evidence demonstrates metformin exerts anticancer effects in various cancers, including the breast (Fan et al. 2015), endometrium, colon, thyroid and esophagus (Moon and Mantzoros 2014), pancreas (Gou et al. 2013; Karnevi et al. 2013), stomach (Kato et al. 2012) and prostate (Kato et al. 2015) by reducing tumor cell growth in part by reducing the activity of mTOR as part of its complex mTORC1 (Pollak 2010; Quinn et al. 2013; Pollak 2012; Pierotti et al. 2013). Therefore, we can hypothesize that in malignancies where the mTORC1 pathway is frequently hyperactivated, such as HNSCC, targeting mTOR with metformin may represent an effective, safe, and low cost therapeutic strategy for cancer prevention.

Metformin showed a striking impact in tumor progression by a significant decrease the number of low and high grade dysplasia in premalignant oral cancer mouse model induced by chemical carcinogen 4NQO (Vitale-Cross et al. 2012). We also found that metformin decreases mTOR activity and HNSCC progression by acting on HNSCC-initiating cells directly (Madera et al. 2015). This result was achieved with metformin concentrations in blood similar to that reported for diabetic patients taking metformin for type 2 diabetes (Balan et al. 2001; Sambol et al. 1996). Remarkably, aligned with our findings two recent large retrospective population case-control cohort studies involving together more than 300,000 diabetic patients demonstrated a decreased OSCC risk in patients on metformin (Tseng 2016; Yen et al. 2015).

Collectively, our findings and the emerging epidemiological data have provided a strong rationale for the future implementation of molecular-targeted chemopreventive trials in HNSCC, using agents impinging on the mTOR pathway with a better safety profile than direct mTOR inhibitors. Indeed, our team has developed a multi institutional phase IIa single-arm, open-label trial (metformin for oral cancer prevention, M4OC-Prevent) in individuals with OPL (NCT02581137; Table 7.2). We hope that the outcome of this first study evaluating the chemopreventive potential of metformin in OPL will pave the way for future mechanism-based precision therapies for HNSCC prevention in at risk patient populations, as well as for the treatment of HNSCC associated with traditional risk factors and the increasing incidence of HPV-associated oropharyngeal HNSCC. Overall, we can expect that the elucidation of the molecular mechanisms driving the growth, survival, and invasive potential of HNSCC cells will provide an opportunity for precision medicine approaches to prevent HNSCC from arising from its potential premalignant precursors and to halt the progression HNSCC lesions, thus increasing the quality of life and life expectancy of HNSCC patients.

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Chapter 8
Survivin as a Therapeutic Target in Squamous Cell Carcinoma

Zakir Khan

Abstract Survivin is a universal tumor marker that belongs to inhibitor of apoptosis protein (IAP) family. It is involved in the control of cell cycle and inhibition of apoptosis. As like other cancers, most of the SCCs also overexpress survivin gene. Tumors overexpressing survivin generally bear a poor prognosis and are associated with resistance to therapy. The differential expression of survivin in cancer versus normal tissues makes it a useful tool in cancer diagnosis and a promising therapeutic target. Disruption of the survivin induction pathway has resulted in an increase in apoptosis and inhibition of tumor growth. In this chapter, we will discuss recent advances in cancer therapeutics targeting survivin gene.

8.1 Introduction

Squamous cell carcinoma (SCC) is an epithelial malignancy that occurs in organs that are normally covered with squamous epithelium which includes skin, lips, mouth, esophagus, urinary tract, prostate, lungs, vagina, and cervix. Given the range of tissues in which it arises, SCC represents the most common cancer capable of metastatic spread in the USA and worldwide (Marinkovich 2007). Despite sharing the name, the SCCs of different body sites form highly diverse group of cancers which can show tremendous differences in their presenting symptoms, natural history, prognosis, and response to treatment. Majority of SCC cases come from non-melanoma skin cancer, head and neck cancer, esophageal cancer, and non-small cell lung cancer. The risk of SCC is strongly linked with environmental factors, such as sun exposure, viral infections and tobacco use (Ridky 2007; Johnson et al. 2008; Rothenberg and Ellisen 2012). SCCs are associated with a high risk of recurrence, resulting in significant mortality and often requiring specialized

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_8

surgical techniques for complete excision. SCC is a highly invasive malignant neoplasm associated with a high risk of metastasis and morbidity (Veness 2007; Rothenberg and Ellisen 2012). It often invades neighboring tissues, and can also metastasize to the lymph nodes, lung and other distant sites.

Despite advances in diagnostic methods and combined treatment modalities, the survival rate of SCC patients has not improved significantly over the last 30 years (Agada et al. 2009) due in part to a lack of reliable early diagnostic biomarkers and a limited number of molecularly targeted therapeutic strategies.

In recent years, apoptosis has become a basic tool in developing cancer research and establishing new cancer strategies. Evasion of apoptosis is one of the common hallmarks of human cancers (Hanahan and Weinberg, 2011). Apoptosis, or programmed cell death , is a genetically controlled process, which maintains developmental morphogenesis and homeostasis of differentiated organisms by removing senescent, unneeded, or dangerous cells (Vaux et al. 1994). Aberrations of this process leading to aberrantly reduced cell death are thought to participate in cancer by promoting increased resistance to therapy and favoring the insurgence of transforming mutations (Khan and Bisen 2013).

Considerable interest has recently focused on the identification of regulators of apoptosis, which may potentially influence the cell death/cell viability balance in cancer. In addition to pro- and anti-apoptotic bcl-2 molecules (Reed 1998), a second gene family of inhibitor of apoptosis (IAP) has been identified (Crook et al. 1993; Yang and Li 2000). Highly evolutionarily conserved from viruses to mammalian cells (Ambrosini et al. 1997), IAP proteins target a downstream step in apoptosis by inhibiting the terminal effector caspase-3 and -7 (Deveraux et al. 1997, 1998; Tamm et al. 1998), and by interfering with processing/activation of the procaspases. In this chapter we will review the therapeutic potential of a IAP family protein survivin in cancer with special reference to SCC.

8.2 Survivin

Survivin is a unique member of the IAP protein family. It is expressed in most of the human tumors, but can barely be detected in the terminally differentiated normal cells/tissues. Molecular mechanisms of regulation of survivin in cancer are not clearly under-stood. Nevertheless, the functional loss of wild-type p53 is often associated with upregulation of survivin (Mirza et al. 2002). Overexpression of survivin generally associated with poor prognosis and resistance to cancer therapy. A growing body of literature suggests nuclear expression of survivin as a good prognostic marker in several cancers. Down-regulation of survivin induces cancer cell apoptosis, and suppresses tumor growth.

Unlike other inhibitor of apoptosis protein (IAPs), survivin is small and has only a single N-terminal BIR domain, a long C-terminal α-helix coiled region, and forms a stable dimer in solution. The BIR domain is thought to be critical for anti-apoptotic function, whereas the coiled domain probably interacts with tubulin

structures (Wheatley and McNeish 2005; Li et al. 1998; Sah et al. 2006). The BIR domain consists of 70 amino acids, which are evolutionarily highly conserved. The survivin "apoptosis inhibitor 4" has its structural homology with the murine TIAP and deterin of *Drosophila melanogaster*; however, the func-tional aspects are only partially unique. Similarly, the genomes of *Xenopus laevis, Xenopus tropicalis*, zebra fish, fugu pufferfish, and rainbow trout encode two different survivin genes (Su1 and Su2). The human survivin gene, spanning 14.7 kb on the telomeric position of chromosome 17, contains four exons and three introns and produces a 16.5 kDa protein (Verdicia et al. 2000; Khan et al. 2006).

Several splice variants of survivin have been identified. The isoforms form almost the full length of survivin, survivin-2B, and survivin-DEx-3 with insertion and deletion of some of the coding and noncoding sequences. The full-length survivin gene consists of a three-intron, four-exon structure. The survivin-2B transcript resulted in the retention of a portion of intron 2, whereas removal of exon 3 was seen in the survivin-DEx-3 transcript. Survivin-3B results from insertion of novel exon 3B, leading to frameshift and premature termination of the protein (Sah et al. 2006, Caldas et al. 2005). The sequence alterations produced from the splice variants markedly change the corresponding protein conformations and subsequently create differences in their ability to inhibit apoptosis. Insertion of exon 2B in survivin-2B, in the essential BIR domain, may lead to a decrease in apoptotic activity. The removal of exon 3 in survivin-DEx-3 likewise interrupts the BIR domain and retains its ability to suppress apoptosis. In addition, loss of this exon results in a frameshift in exon 4, generating a novel COOH-terminal. There are also different subcellular localizations of survivin and its splice variants. Survivin and survivin-2B are predominantly cytoplasmic, whereas survivin-DEx-3 is primarily nuclear (Mahotka et al. 2002; Knauer et al. 2007). The different isoforms of survivin and their localizations in the cell make a regulatory balance between apoptosis and inhibition of apoptosis.

8.3 Functions of Survivin

Survivin has two main functions: one as a chromosomal passenger protein and the other as an inhibitor of apoptosis. Survivin-2B has been shown to be a pro-apoptotic protein that sensitizes resistant leukemia cells to chemotherapy in a p53-dependent fashion. Survivin-Ex-3 functions as an anti-apoptotic protein and is upregulated in malignancies (Mahotka et al. 2002; Sah et al. 2006). Survivin is critical for global normal embryonic development as demonstrated by the early embryonic lethality of mice with homozygous deletions in the survivin gene locus (Wu et al. 2009). Survivin proteins are virtually absent from most normal differentiated tissues; however, these proteins are expressed in certain highly proliferative areas within normal tissues. In contrast, survivin is highly expressed in the majority of human malignancies, derived from different cell origins.

8.3.1 Role of Survivin in Cell Division

Survivin plays important role in the regulation of mitosis (Altieri 2003a). Survivin gene expressed in cell cycle dependent manner, and is only expressed in the G2-M phase. It is known that survivin localizes to the mitotic spindle by interaction with tubulin during mitosis, indicating its involvement in the regulation of mitosis. It is now very well documented that survivin controls multiple facets of cell division in association with other proteins. The essential role of survivin at cell division has been linked to centrosomal function (Li et al. 1998), metaphase and anaphase microtubule assembly (Giodini et al. 2002), and spindle checkpoint regulation. Depletion of survivin caused defects in cell division, such as arrest of DNA synthesis due to activation of the tumor suppressor protein p53 (Altieri 2003a, b). During anaphase of mitosis in survivin-deficient cells, sister chromatids disjoined normally, but one or more of the sister chromatids frequently lagged behind the main mass of segregating chromosomes, probably because of merotelic kinetochore attachments. Survivin-deficient cells initiated but failed to complete cytokinesis, apparently because the spindle midzone and midbody microtubules were absent during late mitosis (Caldas et al. 2005; Vivek et al. 2011). The abnormalities of both chromosome segregation and cytokinesis could be attributed to a defect in the chromosomal passenger protein complex, with a consequent mislocalization of the kinesin-like motor protein MKLP-1 associated with the microtubule abnormalities.

The RNA interference studies on the depletion of aurora B recapitulated the importance of survivin in the proliferation of normal human cells by virtue of its contributions to accurate sister chromatid segregation and assembly/stabilization of microtubules in late mitosis (Altieri 2003a). It has been demonstrated that survivin interacts with Aurora B and inner centromere protein (INCENP). This complex of AuroraB/INCENP/Survivin binds centromere of metaphase chromosomes at the central spindle midzone at the anaphase chromosome, which is characteristic of chromosomal passenger proteins that participate in chromosomal segregation and cytokinesis. Targeting survivin results in aberrant mitotic progression, leading to failed cytokinesis and multinucleation (Uren et al. 2000; Skoufias et al. 2000). Similar results reported in other organisms. A homolog of survivin, Bir1P/Cut17P/Pbh1p in fission yeast interacts with the chromosomal passenger protein (INCENP), Pic1P and the replication initiation factor Psf2P in the course of regulation of chromosomal segregation (Huang et al. 2005).

8.3.2 Role of Survivin in Apoptosis

There are two major types of stimuli, extrinsic and intrinsic, that cause programmed cell death. External factors may act through initiation of ligation of death receptors (CD-95/fas receptor, TNFα-Receptors) leading to activation of initiator caspase-8

(Scaffidi et al. 1998). Intrinsic factors or intracellular stimuli, such as DNA damage, may act via mitochondrial apoptotic pathway initiated by the release of cyt-c and Smac/DIABLO (Deveraux et al. 1998). These proteins activate initiator caspase-9 that mediates apoptosome formation. In general, mammalian IAPs intercept apoptosis through direct or indirect inhibition of initiator caspase-9 or terminal effector caspases (Khan and Bisen 2013, Fig. 1).

Survivin occurs abundantly in the mitochondrial pool, especially in inter mitochondrial membrane space. In mammalian cells, the mitochondrion has emerged as a central region of apoptosis, eliciting arrays of cell death regulators. The mechanism(s) by which survivin, and possibly other IAPs, localizes to mitochondria is currently not known. Conversely, survivin was not present in mitochondrial fractions of nor-mal tissues, which suggests that its localization to mitochondria may preferentially, or exclusively, be associated with oncogenic transformation. An attractive candidate for this pathway is the molecular chaperone Hsp90 (Young et al. 2003), which participates in mitochondrial import of client proteins and associates with survivin and other IAPs in vivo.

Several mechanisms have been proposed to explain anti-apoptotic activity of survivin. Direct suppression of caspase-3 by survivin has also been speculated by some investigators; yet, survivin lacks structural components present in other IAPs that allow their direct binding to caspase-3. Phosphorylation of survivin on the threonine at position 34 (Thr34) is critical for a functional survivin molecule. Survivin may indirectly inhibit caspases via intermediate proteins. Its ability to associate with caspase-9 and Smac/DIABLO indicates that it may inhibit the intrinsic pathway of apoptosis by interfering with post-mitochondrial events (Tamm et al. 1998). Smac/DIABLO may act as a pro-apoptotic protein through its participation in the activation of caspase-9 (Apoptosome formation) and binds IAPs, and thus prevents them from inhibiting caspases. Survivin has affinity with Smac/DIABLO, so it may inhibit apoptosis through antagonizing the pro-apoptotic ability of Smac/DIABLO (Song et al. 2003, Khan and Bisen 2013). A point mutation at Asp-71 in survivin results in the failure of survivin to complex with Smac/DIABLO. Consequently, this was shown to abolish survivin's ability to protect against cancers. XIAP is a strong inhibitor of apoptosis, interacts directly with caspases, and inhibits them. Smac/DIABLO is the negative regulator of XIAP. Survivin may interact with Smac/DIABLO, which ultimately leads to inhibition of apoptosis (Fig. 8.1).

8.4 Survivin Expression in SCC: Prognosis and Diagnosis

Strong survivin expression is observed in the vast majority of cancers (Fukuda and Louis 2006). These include head and neck, esophageal, lung, ovarian, central nervous system, breast, colorectal, bladder, gastric, prostate, pancreatic, laryngeal, uterine, hepatocellular, and renal cancers, as well as melanoma and soft tissue sarcomas. Almost all SSCs also expressed high level of survivin. Retrospective

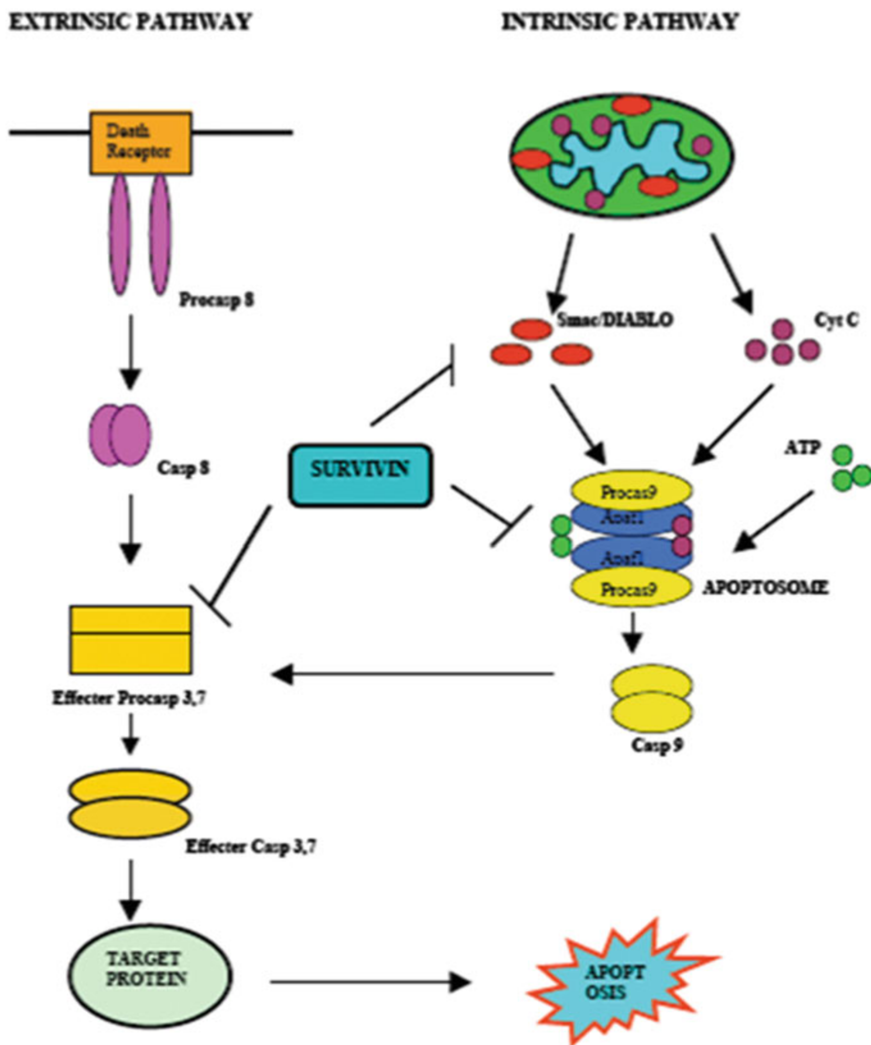

Fig. 8.1 Apoptotic pathways and the site of survivin anti-apoptotic action. Intrinsic and extrinsic apoptotic pathways are shown that coverage into a common downstream pathway of effecter caspase activation. In intrinsic pathway Cyt-c released from mitochondria in response to several stresses. Cyt-c interacts with Apaf-1 and procaspase-9 in presence of dATP to form apoptosome complex that leads to activation of procaspase-9. Active caspase-9 activates effector caspases-3, 7, and 6, which in turn induce apoptosis. Survivin most probably blocks, directly and/or indirectly, caspase 9 activation. It may directly inhibit initiator caspase-9 and effector caspases-3, 7. Smac/DIABLO is a pro-apoptotic protein that inhibits activity of IAPs such as XIAP. Survivin antagonize the activity of Smac/DIABLO and may help in the action of another IAPs

studies have evaluated the correlation between survivin, disease variables, and clinical outcomes. Elevated survivin expression is associated with clinicopathologic variables of aggressive disease and shows a strong correlation with shorter disease-free or overall survival in most studies, identifying it as a significant independent prognostic indicator of poor outcome in patients with most SCC types (Table 8.1). In cancer cells, high level of survivin is commonly associated with increased proliferation, reduced levels of apoptosis, resistance to chemo-radiotherapy, and increased rate of tumor recurrence (Khan et al. 2009, 2010a, b, 2012; Zhu et al. 2011).

Table 8.1 Survivin expression in SCCs

Type of SCC	Method	Correlation with survivin			References
		Clinicopathologic variables	Poor prognosis	Survival	
Oral	IHC, RT-PCR, WB (110) IHC (78) IHC, RT-PCR (29)	NC Aggression, invasion	Prognostic marker	↓	Lo Muzio et al. (2003) Lo Muzio et al. (2005) Khan et al. (2009)
Laryngeal	IHC (68) IHC (86) IHC (102)	Site Metastasis Metastasis	Prognostic marker	↓	Pizem et al. (2004) Chen et al. (2004) Dong et al. (2002)
Esophageal	IHC (51) RT-PCR (57) IHC (84)	Nodal status NC Metastasis	Prognostic marker	↓	Kato et al. (2001) Ikeguchi and Kaibara (2002) Grabowski et al. (2003)
Cervical	IHC (17) IHC, WB (53)	NC	Prognostic marker	↓	Yoshida et al. (2003) Lee et al. (2005)
Skin	IHC (89) IHC, WB	Size, nodal metastasis Poor differentiation and invasion	NC	↓	Muzio et al. (2001) Dallaglio et al. (2014)
Non-small cell lung cancer	IHC (58), RT-PCR (83) Meta-analysis (2703)	NC	Prognostic marker	↓	Atikcan et al. (2006) Falleni et al. (2003) Zhang et al. (2012)

Survivin is one of the few proteins, which is differentially expressed in tumor cells as compared to most normal tissues. This characteristic enables it to be a potential marker of cancer. The dual function of survivin indicates that it is expressed in tumor cells associated with growth and survival advantages resulting in tumor onset and progression. An increasing number of original publications suggest that nuclear expression of survivin may be a better and more reliable prognostic marker (Dong et al. 2002; Falleni et al. 2003; Grabowski et al. 2003; Muzio et al. 2001; Dallaglio et al. 2014). An increased level of survivin is a powerful negative prognostic marker for tumors. It can be detected in tumor specimen by immunohistochemical methods, which is a quick prognostic indicator for identifying patients at risk of recurrence of the disease and those who would be benefited from more aggressive follow up and alternative protocols (Table 8.1). In several SCCs, survivin provide a potential evaluation indices for early diagnosis and prognosis (Takeno et al. 2010; Lu et al. 2012). In a recent study to be presented at the AACR Annual Meeting 2014 on April 8 by Dr. Ranee Mehra, a medical oncologist who specializes in head and neck cancers at Fox Chase Cancer Center, and colleagues, showed a correlation between the expression levels of survivin and other proteins in HNSCC negative for human papillomavirus (HPV). These tumors have a poorer prognosis than HPV-positive HNSCC. This study proved survivin as a marker for improved survival, especially in patients who were treated with surgery plus radiation. Tumors with less than a median level of survivin expression were associated with improved patient survival compared to tumors with more than a median level of survivin (Mehra et al. 2013; Mehra 2014).

Survivin or its autoantibody also present in biological fluids of cancer patients could provide them a potential diagnostic tool. In case of bladder cancer, survivin has been detected in the urine of the patients, which provides a specific and sensitive diagnostic marker. In OSCC, survivin detected in patient's saliva, which could be an interesting diagnostic tool (Santarelli et al. 2013). In addition, anti-survivin antibodies circulate in cancer patients; which may be used as a diagnostic tool (Altieri 2001). Recently, survivin auto-antibodies found in the saliva of OSCC patients. This study provides a novel and practicable approach for OSCC screening using salivary auto-antibodies as a diagnosis tool (Chih-Ching et al. 2014). Several companies have now come up with diagnostic kits for early detection of cancer, which are largely based on survivin. For example, Biocore Pvt. Ltd has developed a kit for quantification of human survivin from cell lysates by colorimetric immunoassay. Novus Biologicals (Cat. BEK-2121-2P) and Cell signaling technology (Cat. 877-616-CELL-2355) have also developed human Survivin ELISA Kit for cancer detection.

8.5 Therapeutic Targeting of Survivin

As we described above, survivin is a critical regulator of multiple cellular processes, including proliferation and apoptosis, and its expression appears to be a consistent feature of hyper-proliferative lesions contributing to the formation of hyperplasia. It

is expressed in the G2/M phase of the cell cycle to support rapidly dividing cell machinery (Li et al. 1998), and helps in proper segregation of chromosomes during cell division (Kaitan et al. 2000; Lens et al. 2006). Several techniques have been developed to examine therapeutic potential of susvivin in cancer treatment. These include suppressing survivin expression by antisense, ribozyme, siRNA, or shRNA approaches or antagonizing survivin function by dominant-negative survivin or by Cdk inhibitors. Anti-survivin therapy has been evaluated in several preclinical models using mice harboring pre-established tumors.

8.5.1 Antisense Technology

Antisense technology, with its potential to selectively control gene expression and cellular phenotype, is proving useful as a therapeutic application. Several types of antisense approaches (viz. antisense oligonucleotides, small interfering RNA, and short-hairpin RNA) can be used to inhibit expression of target genes. These interfering RNAs can be chemically synthesized and transfected into cells or directly expressed intracellularly from a plasmid DNA or a viral vector.

Down-regulation of survivin by a targeted antisense oligonucleotide could be potential gene therapy to combat several cancers. Transfection of cancer cells with antisense of survivin enhances sensitivity of tumor cells to chemotherapy and radiotherapy.

Several anti-survivin oligonucleotides have been tested for their ability to block survivin expression in many types of cancer cells. In earlier study, Olie et al. (2000) tested many anti-survivin oligonucleotides in lung carcinoma. Out of several efficient oligonucleotides, 4003 was found to be the most effective in growth inhibition and apoptosis in lung carcinoma cell lines. Our research involves in understanding the role of survivin in HNSCC resistance to conventional drugs. We have demonstrated that survivin overexpression in HNSCC cells provide resistance against conventional drugs including chemotherapy and radiotherapy. We used siRNA technique for inhibiting survivin expression in cancer cells, which significantly inhibits cancer cell proliferation and sensitized them to chemotherapy (cisplatin and paclitaxel) and radiotherapy (Khan et al. 2010a, b). For pre-clinical studies, we developed lentivirus vector to deliver survivin-siRNA (Khan et al. 2012). A significant reduction in HNSCC tumor development was observed in pre-clinical studies using lentivirus siRNA therapy. Further, a high efficacy was observed when we used combination of lentivirus therapy and chemotherapy or radiotherapy (data not published). Many other in vitro and in vivo studies have been conducted, in which anti-survivin method used either alone or in combination with conventional drugs to control cancer cell proliferation, including most of the SCCs, oral (Scheper et al. 2007; Su et al. 2010), Laryngeal (Li et al. 2011), head and neck (Sun et al. 2012), skin (Dallaglio et al. 2012, 2014), esophageal (Wang et al. 2005),

Lung (Chen et al. 2012). Wen et al. (2013) investigated inhibitory function of survivin in laryngeal SCC cell lines GRIM-19 and Hep-2 using plasmid-based survivin-specific short hairpin RNA. Proliferation of laryngeal cancer cell lines undergoing transfection with p-siRNA survivin was markedly inhibited (79%). Silencing of survivin also significantly inhibit the growth and induce the apoptosis of Hep-2 in vivo. Recently, Stoleriu et al. (2014) successfully tested multimodality therapy regimen to treat chemoresistant NSCLC cell lines. siRNA-mediated silencing of survivin along with other genes in these cell lines sensitized them to chemotherapies and significantly induced apoptosis. Survivin is overexpressed in OSCC, making it a promising target for gene therapy. In a study, lentivirus vector encoding shRNA targeting survivin was used to suppress survivin gene in vitro and in vivo (Jiang et al. 2006). Inhibition of survivin reduced proliferation of tumor cells in vitro and sensitized cells to radiation and vincristine. In the OSCC xenograft model, the development of tumors as well as the growth of established tumors was inhibited by transfection of lentivirus. Similar growth inhibitory effects of survivin have been observed by using survivin siRNA in esophageal (Wang et al. 2005) and skin SCC tumor xenographt models (Dallaglio et al. 2014).

As like in SCCs, inhibition of survivin showed anti-proliferative effects in many other malignancies. Most of the mesothelioma cancer cells underwent apoptosis and were more sensitive to chemotherapy and radiotherapy when treated with 20-mer phosphorothioate antisense oligonucleotide targeting nucleotides 232–251 of survivin mRNA (Chuunyao et al. 2002). Growth of lymphoma cell lines was significantly inhibited by using antisense oligonucleotide (ASOND) (Ansell et al. 2004). Treatment of colon cancer by adenoviral antisense vectors (pAd-CMV-SAS) resulted in an increase of the Go/G1 phase population in the cell cycle and increased their sensitivity to chemotherapeutic drugs in vitro (Yamamoto et al. 2003). In PC-3 prostate cancer cell treatment with antisense survivin cDNA caused nuclear fragmentation, hypodiploidy, cleavage of a 32 kDa proform caspase-3 to active caspase-3, and proteolysis of the caspase substrate poly(ADP-ribose) polymerase (Mara et al. 2003). In another study, a similar observation has been made with human neuroblastoma cell line SK-N-MC (Guan et al. 2002). Transfection of cancer cell lines by adenoviral vector harboring a tandem type siRNA expression unit targeting survivin resulted in gene knockdown and induced apoptosis (Ling and Li 2004; Uchida et al. 2004). The transfection of cancer cells with Adv-siSurv showed significantly attenuated growth potential, both in vitro and in vivo. Moreover, intratumoral injection of Adv-siSurv significantly suppressed tumor growth in a xenograft model using U251 glioma cells. A shRNA, containing two 20–21 bp reverse repeat motifs of a survivin target sequence with a 4–8 bp spacer, effectively downregulates expression survivin in liver cancer cell lines Hep G2 and SMMC-7721, after transfection (Yang et al. 2003). These findings suggest that targeting of survivin using antisense technology may have a potential role in the selective therapy of cancer.

8.5.2 Dominant Negative Constructs

In this technology, an essential amino acid of the protein is replaced by another amino acid, which leads to the loss of function, but this nonfunctional protein has the same target as normal protein. Dominant negative mutants compete with normal proteins for their target and suppress the function of normal proteins. Several dominant negative constructs have been discovered for survivin, from which the T34A mutant of survivin is best known. Transduction of lung, breast, cervical, prostate, colorectal, liver, and skin cancer cell lines with pAd-T34A (replication-deficient adenovirus vector encoding a nonphosphorylated The 34Ala mutant survivin) increased cyt-c release from mitochondria, processing caspase-3 to the active sub-units of approximately 17 and 19 kDa increased caspase-3 catalytic activity, and facilitated tumor cell apoptosis induced by taxol and adriamycin anticancer drugs (Mesri et al. 2001). In malignant HeLa cells, transfection with survivin mutant (survivin-N and survivin-T34A) could partially reverse the malignancy of HeLa cells (Zhu et al. 2003). In a breast cancer model in mice, intratumor injection of pAd-T34A produced significant reduction of pre-established tumor size with an increase in apoptotic cells (Mesri et al. 2001). T34A-survivin injection into disseminated breast cancer cells in the peritoneal cavity of severe combined immunodeficient mice significantly reduced tumor growth. Interestingly, this therapy did not affect cell viability normal cells and no systemic toxicity was noted in mice treated with pAd-T34A, suggesting that targeting survivin by adenovirus may provide selectivity for tumor cells and limited toxicity for normal tissues. Intratumoral injection of pAd-T34A into prostate cancers in mice significantly inhibited tumor growth (Hayashi et al. 2005) and enhanced anti-androgen sensitivity (Zhang et al. 2005).

The efficacy of negation of anti-apoptotic activity of survivin was also demonstrated in gastric cancer cell lines (BCG-823 and MKN-45) through decreased cell growth and increased rate of spontaneous apoptosis, by introducing a plasmid construct expressing a dominant negative mutant with a cysteine residue at amino acid 84 replaced with alanine (C84A) (Shui et al. 2003). In the PC-3 prostate cancer cell line, transfection with C84A mutant was sufficient to visualize all biochemical hallmarks of apoptosis, including hypoploid DNA content, caspase-3 activities, and cleavage of caspase substrates. In cutaneous SCC cell lines, C84A resulted in spontaneous apoptosis in the absence of other genotoxic stimuli. This was associated with a five-fold increase in the sub-G0/G1 fraction corresponding to apoptotic cells and a decrease in proliferating cells with 4N DNA content (Grossman et al. 1999). Injection of dominant-negative C84A-survivin in adeno-associated virus into colon cancers inhibited tumor cell growth and angiogenesis in mice without obvious organ toxicity (Tu et al. 2005). In large-cell lymphomas, injection of survivin mutant C84A reduced tumor cell growth and enhanced tumor-specific Cytotoxic T lymphocytes-mediated cell death (Kanwar et al. 2001).

Treatments of mice bearing lung carcinoma with survivin T34A construct suppressed tumor growth. Moreover, the anti-tumor effect of T34A combined with

radiation was greater than their additive effect when compared with the expected effect of the combined treatment. These results suggest that inhibition of survivin using a survivin T34A dominant-negative mutant could sensitize lung SCC cells to radiation efficiently (Yuan et al. 2010). In another study, lyposome complex was used to deliver survivin T34A mutant with or without cisplatin. This treatment significantly sensitized lung SCC cells to cisplatin drugs. In mice, tumor volume reduced significantly when treated with intravenous injections of lyposome-T34A. Moreover, the antitumor effect of lyposome-T34A combination with cisplatin was greater than their anticipated additive effects, suggesting synergistic interaction. In vivo studies also showed anti-angiogenesis effects of survivin dominant negative constructs (Yu et al. 2010; Xu et al. 2012). For understanding whether survivin double point mutant could achieve more potent inhibitory effect on the growth, Zhang et al. (2008) transduced hepatocellular cancer cells with adenoviruses expressing survivin double mutants (Ad-T34A-C84A). Study showed much stronger cancer cell killing with Ad-T34A-C84A as compared to survivin mutants T34A or C84A alone.

8.5.3 Ribozyme Technique

Ribozyme (ribonucleic acid enzyme) is a powerful new therapeutic tool used to diminish RNA molecules in cells (Sullivan 1994; Johnston et al. 2001). The hammerhead ribozyme is the smallest and best characterized (Pley et al. 1994). All hammerhead ribozymes contain three base-paired stems and a highly conserved core of residues required for cleavage. Hammerhead ribozymes cleave after NUH (where N can be any nucleotide, and H can be any nucleotide except G) sequences of target mRNA to suppress the target protein expression (Haseloff and Gerlach 1988; Wochner et al. 2011). Ribozyme specificity is determined by the paired regions flanking the cleavage site. Cleavage of mRNA with ribozyme is significantly more stringent than short interfering RNA approaches, which often produce off-target effects.

Several ribozymes has been developed to inhibit survivin in cancer cells. Survivin-specific mRNA undergoes cleavage due to ribozyme treatment resulting in inhibition of translation and consequent retardation in tumor growth. Choi et al. (2003) has been designed two hammerhead ribozymes, viz., RZ1 and RZ2, targeting human survivin mRNA. These ribozymes efficiently cleaved the human survivin mRNA at nucleotide positions +279 and +289. For functional study, breast cancer cell line (MCF-7) was transduced with adenoviral vector encoding these ribozymes, which resulted in a significant reduction of survivin mRNA as well as protein, and consequently induced apoptosis (Choi et al. 2003). Infected prostate cancer cell lines by the adenoviral vector that encodes a ribozyme targeting the 3′ end of the survivin mRNA increased susceptibility of PC-3 and DU145 cancer cells to apoptosis (Pennati et al. 2004). In another study, transduction of melanoma cell lines JR8 and M14 with a vector-carrying ribozyme sequence led to a decrease of

the survivin level and sensitized cancer cells to radiotherapy and chemotherapy (Pennati et al. 2003, 2004). Four hammerhead ribozyme (R1–R4) to suppress survivin gene has been designed by Fei et al. (2008). Adenoviruses encoding these ribozymes tested for control cancer cell proliferation in vitro and for in vivo tumor growth suppression. Suppression of survivin expression induced mitotic catastrophe and caspase-3-dependent cell death. Administration of the ribozyme adenoviruses inhibited tumor growth in a hepatocellular carcinoma xenograft mouse model. Interestingly, co-expression of R1, R3 and R4 ribozymes has been tested, which synergistically suppressed survivin and, as this combination targets all major forms of the survivin transcripts, produced the most potent anti-cancer effects (Fei et al. 2008). Despite high substrate cleavage efficiency, clinical application of ribozyme still limited due to misfolding and RNA degradation of ribozyme, when fused to a carrier. Liu et al. (2007) constructed a chimeric ribozyme escorted by the motor pRNA of bacteriophage phi29 to achieve proper folding and enhanced stability. This chimeric ribozyme has been found to suppress survivin mRNA and protein efficiently, and induced apoptosis in a variety of cancer cell lines including nasopharyngeal, lung, cervical, breast and prostate without causing significant non-specific cytotoxicity. These studies using in vitro and in vivo anti-survivin therapies clearly indicate that disrupting survivin in cancers may be clinically beneficial.

8.5.4 Histone Deacetylase Inhibitors

Histone acetylation/deacetylation plays an important role in epigenetic transcrip-tional regulation in eukaryotic cells (Grunstein 1997). Histone acetyl-transferases (HATs) are recruited by transcription factors and are associated with activation of transcription, whereas histone deacetylases (HDACs) are involved in transcriptional silencing. Histone acetylation is tightly controlled by the dynamic equilibrium between competing HATs and HDACs (Grunstein 1997; Hassing and Schreiber 1999). The HATs bring about transcriptional activation by the addition of acetyl groups on the amino group of lysine residues in the NH2-terminal tails of core histones, and HDACs reverse this reaction by removing the acetyl groups from the acetylated lysines in histones, and thereby bringing transcriptional (Hassing and Schreiber 1999; Kouzarides 1999; Jenuwein and Allis 2001). Abnormality in these enzymes can disturb the expression of several apoptotic and cell cycle regulatory genes, including survivin. Inhibition of HDACs is emerging as a new strategy in human cancer therapy. Several drugs have been discovered, which specifically inhibit the activity of HDAC. An important drug, clamydocin, used as a potent inhibitor of cell proliferation, is a potent inhibitor of HDAC (De Schepper et al. 2003).

Like other HDAC inhibitors, clamydocin induces the expression of hyper-acetylated histones H3 and H4 in cancer cells, increases the expression of p21, and causes an accumulation of cells in the G2/M phase of the cell cycle. In addition,

clamydicin induces apoptosis by activating caspase-3, which in turn leads to the cleavage of p21 and drives cells from growth arrest into apoptosis. Clamydocin also decreases the level of survivin, which is mediated by proteasome-mediated degradation. LAQ824, another inhibitor of HDAC, also down-regulates the levels of survivin and induces apoptosis of cancer cells (Fei et al. 2004).

8.6 Immunotherapy

Survivin has generated much enthusiasm to be leveraged as an agent for cancer immunotherapy, because of its exclusive expression in cancer cells. The antigens expressed by tumor cells can be recognized by the host immune system. The immune system recognizes antigens in the form of short peptides binding to the major histocompatibility complex (MHC) molecules. Survivin evidently induces activation of T-cell antigen. Activating the body's immune system to deal with tumor cells has been a long-standing goal of cancer therapeutics (Gold et al. 1997). It has been discovered that the immune response against survivin-derived epitopes can be induced (Reker et al. 2004). Several survivin-derived epitopes have been tested to induce cytotoxic T-lymphocyte (CTL) activity against tumor cells.

The peptides derived from survivin protein can generate an immunogenic response, which lead to designing experiments in which autologous dendritic cells are infected with survivin-expressing recombinant adenovirus. Such interaction was performed with an expectation that some of the endogenously processed peptides from survivin could be displayed in immunogenic forms on HLA-A2. The dominant negative mutant of survivin was used in place of wild type survivin in order to avoid any pro-oncogenic side effects (Pisarev et al. 2003). Immunization with these dendritic cells induced a T-cell immune response against three different survivin-derived HLA-A2 matching peptides. Significant CTL activity was found against HLA-A2 positive MCF-7 tumor cells that express survivin (Pisarev et al. 2003). Survivin's potential to induce a cellular T-cell response was demonstrated in an experimental assay in which CTL induced lysis of B-cells transfected to present survivin peptides on its surface (Friedrichs et al. 2006).

Survivin-derived HLA class I restricted T-cell epitopes were reported to induce CTL response in cancer patients (Andersen and Thor 2002; Hirohashi et al. 2002; Reker et al. 2004). Survivin-induced CTC was reported to kill HLA-matched tumors with origin from different tissue types (Schmidt et al. 2003; Siegel et al. 2003). An HLA-24-restricted immunogenic peptide survivin-2B80-88 (AYACNTSTL) has been identified that recognized by $CD8^+$ CTL (Hirohashi et al. 2002). Based on this immunogenic peptide, a vaccination to neutralize survivin-2B splice variant, which is abnormally expressed in various types of tumor tissues and tumor cell lines, was subjected to phase-I trial in patients with advanced or recurrent lung, breast and colorectal cancer (Tetsuhiro et al. 2004). Another Phase I trial has been started recently to evaluate the safety and the efficacy of survivin-2B80-88 peptide vaccination in HLA-A24-positive patients with advanced

or recurrent OSCC patients. The vaccines are giving subcutaneously or intratumorally. Initial results of this clinical trial demonstrated the safety and suggested the marginal clinical effectiveness of the survivin-2B peptide vaccination alone for oral cancer patients (Phase-I UMIN000000976, Miyazaki et al. 2011). However, subsequent clinical trials of survivin-based vaccination in combination with various adjuvant drugs could be a promising therapeutic strategy to tackle advanced cancers.

Survivin-specific antibodies have found in the blood samples of cancer patients (Friedrichs et al. 2006). Importantly, it is observed that survivin antibod-ies were absent in healthy subjects, which proves survivin's ability to elicit full a humoral immune response. The isolation of survivin-specific antibody has also opened the opportunity for rational computer-aided designing of epitopes, which would be consequently used in the development of effective cancer vaccines. The processed survivin was presented on dendritic cells reported to induce specific CTL (Schmitz et al. 2000). Another approach relied on using epitopes present on survivin to detect a specific T-cell response in cancer patients by ELISPOT assay (Andersen et al. 2001a, b). Moreover, the evidence of the presence of survivin-specific T-cells in the blood, and in the tumor lesions, should be regarded as encouraging proof that a survivin-induced T-cell response would be targeted toward tumorous tissues (Andersen et al. 2001b). Dr. Hooijberg group is exploring the possibility of dendritic cell based vaccination targeting survivin for the treatment of HNSCC (Turksma et al. 2013). The research team has shown that survivin-specific T cells can be measured ex vivo in the peripheral blood of patients with HNSCC by tetramer analysis and from the tumor-draining lymph node of a patient with locally advanced breast cancer by ELIspot analysis. Survivin-based vaccines like SurVaxM have entered into clinical trials and are expected to be a promising cancer immunotherapeutic agent (National Cancer Institute trial NCT01250470). The vaccine SurVaxM is a survivin peptide mimetic. These findings suggest that manipulation of the anti-apoptotic survivin pathway may provide a novel approach for the treatment of cancer.

8.7 Conclusion

Survivin is ubiquitously and exclusively expressed in cancerous cells of diverse origin, which makes it an ideal therapeutic and diagnostic/prognostic marker. It is significantly expressed in various cancers, including SCCs. High expression of survivin has been associated with poor survival and chemo—and radioresistance among cancer patients. Functionally, it is known to promote tumorigenesis by inhibiting apoptosis and ensuring cell division among cancer cells. The critical role of survivin in cancer cell proliferation has been established through gene silencing experiments in which selective silencing of survivin has been shown to inhibit tumor growth and increase efficacy of other treatment options. Various therapeutic approaches like immunotherapy, small-molecule inhibition, and gene silencing are

in different clinical/discovery phases; therefore, we can expect survivin-based therapy to be available in the near future. The success of survivin-based therapy will depend on efforts of the scientific community to design a cancer management plan by convergence of diverse approaches targeting survivin, for instance, by effective combination of immunotherapy, targeted drug, and radiotherapy.

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Chapter 9
MicroRNA: Utility as Biomarkers and Therapeutic Targets in Squamous Cell Carcinoma

Daniel W. Lambert, Hataitip Tasena and Paul M. Speight

Abstract In recent years there has been an explosion in understanding of the roles non-coding RNA play in the pathogenesis of malignancy, including in squamous cell carcinomas. The majority of this research effort has focussed on microRNA, a class of small RNA able to regulate the expression of protein coding targets which frequently show aberrant expression patterns in cancer. Owing to their ready detection in bodily fluids, including saliva, interest has grown in their utility as biomarkers for diagnosis, prognostication and monitoring treatment response. In addition, evidence is growing that they may represent viable therapeutic targets. This chapter will summarise the current knowledge of microRNA expression changes in head and neck squamous cell carcinomas and give an overview of the translational opportunities they currently offer.

9.1 MicroRNA: Small but Powerful Regulators of Gene Expression

MicroRNA (miRNA) are defined as short, ~22 nucleotide, RNA molecules which do not act as templates for the translation of a protein product (hence they are part of a large and diverse group of 'non-coding' RNA which also includes ribosomal RNA, transfer RNA and long non-coding RNA, amongst other RNA species). They are predominantly derived from sequential processing of RNA polymerase II-generated precursor transcripts by Drosha/Pasha and the RNase III endonuclease DICER (Fig. 9.1). Genes encoding miRNA are found throughout the genome, but

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© Springer Science+Business Media B.V. 2017

S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_9

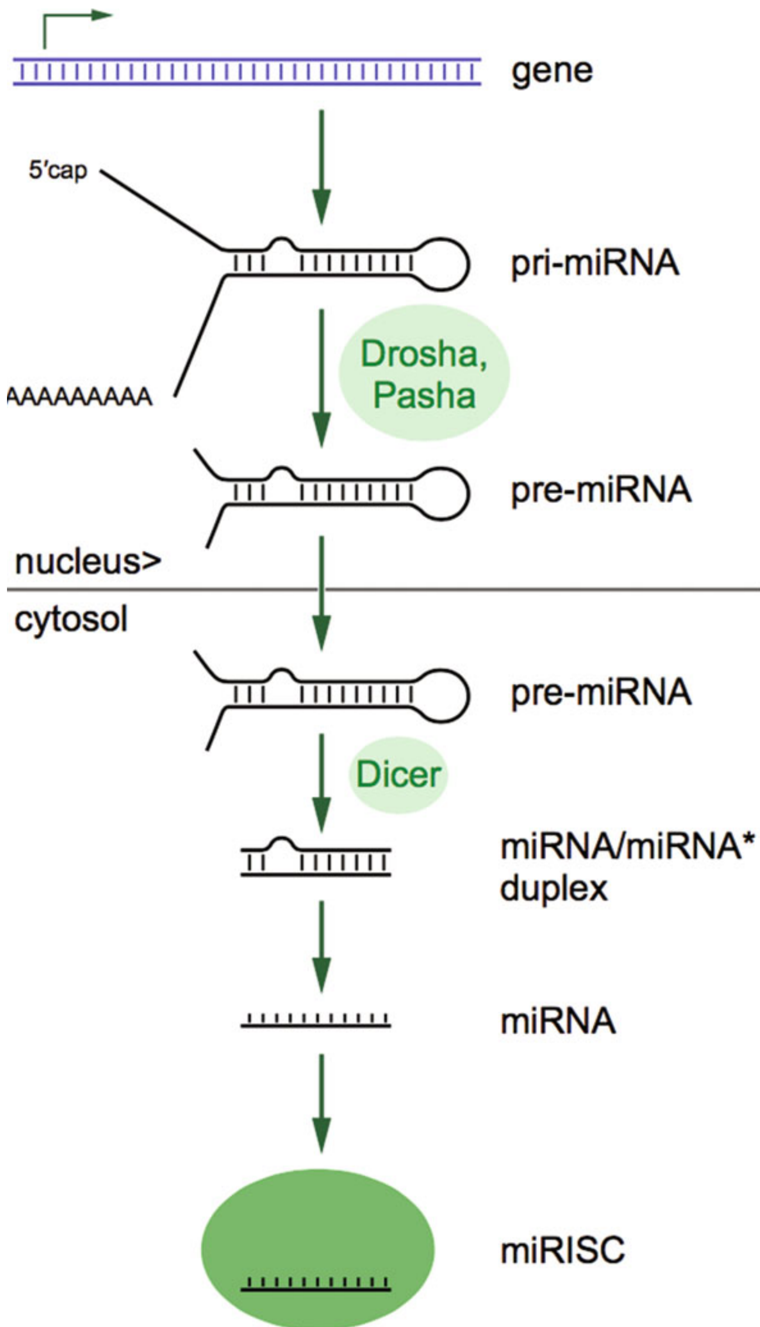

Fig. 9.1 Biogenesis of microRNA. Schematic illustrating the biogenesis of mature miRNA. Source: https://commons.wikimedia.org/wiki/File:MiRNA_processing.svg

are often found associated in clusters, and frequently associate with genomic loci which encode proteins. They may also be generated from intronic regions (so called 'mirtrons'), transfer RNA (tRNA) and small nucleolar RNA (snoRNA) precursors, as well as long non-coding RNA (lncRNA) through a different mechanism (Fig. 9.1). At the time of writing 35,828 mature miRNA products have been annotated (www.mirbase.org), but it is likely the number of functionally active miRNA is considerably lower than this (around 1900 miRNA have been annotated with high confidence across all species analysed).

The ~22 base pair mature miRNA associate with Argonaute (Ago) proteins which form part of the RNA-induced silencing complex (RISC) (Fig. 9.1). The guide strand is retained in the complex and forms Watson-Crick base pairs with complementary 7–8 nucleotide 'seed sequences' present predominantly in the 3′ untranslated region (3′UTR) of target transcripts. This base pairing leads to reduced expression of the protein encoded by the target RNA either by reducing translation by competing with translational machinery or promoting degradation of the transcript by recruiting nucleases following deadenylation of 5′ de-capping. Although the mechanisms dictating the method by which protein production is decreased are not fully understood, the ultimate consequence is repression of expression of the target gene(s) [reviewed in Krol et al. (2010)]. It is thought that over 60% of protein-encoding genes are subject to miRNA-mediated regulation, and a single miRNA may target several hundred transcripts. It is likely, therefore, that miRNA-mediated regulation of gene expression is highly dynamic, depending on contextual cues. In addition, the magnitude of suppression of protein expression by a single miRNA is often mild, leading to the hypothesis that miRNA 'fine tune' gene expression rather than acting as the dominant regulatory mechanism; however, evidence exists to suggest that the miRNA-mediated targeting of multiple components of a pathway responsible for a particular cellular response may collectively have a profound effect on cell behavior; indeed, frequently a single miRNA may target several members of the same pathway, leading to amplification of its effects.

9.2 MicroRNA Alterations in Squamous Cell Carcinomas

It has long been understood that alterations in miRNA expression in malignant cells of epithelial origin, such as squamous cell carcinoma cells, may impact the behavior of the cancer cells and promote tumourigenesis. Frequently, miRNA showing reduced expression in cancer cells have been referred to as tumour suppressor miRNA, and those with increased expression as oncogenic miRNA (or oncomiRs). Whilst it is likely that this is something of an over-simplification, and that specific context and cellular heterogeneity within the tumour will impact upon the function of specific miRNA, a number of miRNA have been identified with tumour suppressive or oncogenic functions. For a comprehensive analysis of aberrant miRNA expression in cancer a number of excellent reviews are available [see e.g. Calin et al. (2006)].

In squamous cell carcinomas, a number of studies have identified widespread alterations in miRNA expression profiles. Some of these have used cell cultures derived from head and neck squamous cell carcinomas (or premalignant lesions), others fresh/frozen tissue, and many have utilized archival, formalin fixed paraffin embedded (FFPE) tissue. Care must be taken in the interpretation of the data obtained from all of these methods; cell cultures may not accurately reflect the tissue of origin, fresh and frozen tissue may suffer from degradation of RNA and RNA extracted from FFPE tissue may be fragmented, all of which may impact on the validity of the findings. The methodology used to profile miRNA expression may also impact upon the data obtained; several methods have been employed including hybridization microarrays, microfluidic tiling low-density arrays (TLDA) and next generation sequencing (miRNA-seq) being the most commonly used. All have advantages and disadvantages that are beyond the scope of this book to cover, but are reviewed in detail elsewhere (Pritchard et al. 2012). An important issue to consider with miRNA expression profiling conducted on surgically resected tissue is the contribution of other cell types to the miRNA expression profile obtained. With these caveats in mind, it is still clear that squamous cell carcinoma cells contain widespread aberrations in miRNA expression profiles which appear to be dependent on the stage of disease and therefore may hold prognostic or diagnostic promise (see Sect. 9.6 for a detailed discussion of this). Indeed, a recent large analysis of data from over 400 patients in The Cancer Genome Atlas (TCGA) identified aberrant expression of a number of miRNA previously reported to be altered in HNSCC using diverse methodologies (Zou et al. 2014).

9.3 Mechanisms of Regulation of miRNA Expression in Squamous Cell Carcinomas

In general, miRNA are subject to the same mechanisms of regulation of expression as protein coding genes, and similar mechanisms underlie their differential expression in cancer cells. For example, aberrant EGFR signalling, a common feature of many cancers including SCCs, is reported to lead to the suppression of expression of the tumour suppressive miR-143/145 cluster. This cluster has also recently been reported to be subject to regulation by the notch signalling pathway, a signalling node known to be disrupted in a large number of SCCs. Both aberrant EGFR and notch signalling lead to excessive proliferation and other protumourigenic features, implicating miR-143/145 in these changes. This, however, also acts as a good example of how sampling artefacts may influence interpretation of miRNA function in cancer, with a compelling report indicating that miR-143/145 are mesenchymal-specific miRNA, and that changes in expression attributed to alterations in cancer cells actually result from differences in the amount of stromal cells present in the tissue analysed.

Another pathway likely to play a key role in the regulation of miRNA expression in SCC cells is that directed by *TP53*. *TP53* is a key tumour suppressor gene which

is frequently mutated or down-regulated in SCC, leading to defects in mechanisms regulating proliferation, apoptosis and metastasis. Mutations in TP53 are strongly correlated with patient survival. The protein product of *TP53*, p53, is a transcription factor reported to regulate a number of miRNA (including miR-145, and the putative oncomiR, miR-34) both directly at the transcriptional level, by interaction with regulatory elements in the gene encoding the miRNA, and at the post-transcriptional level by altering the processing of the precursor miRNA, leading to changes in the amount of the mature, functionally active miRNA.

A family of transcription factors primarily involved in the regulation of organismal development, the HOX genes, are also known to play a role in dysregulating miRNA expression in SCC. HOXD10 was found to be upregulated in cells and tissue isolated from HNSCC and to functionally target miR-146a, a miRNA known to regulate inflammatory responses. In addition, another HOX gene, HOXB9, is over-expressed in HNSCC in concert with miR-196a, a miRNA transcribed from the same gene and possibly derived from the same primary transcript. miR-196a was shown to target a number of genes encoding proteins promoting migration and invasion of cancer cells.

Epigenetic modifications of genomic DNA are also known to contribute to aberrant miRNA expression in a wide range of cancers, including SCCs. These chemical changes, which do not alter the DNA sequence but are heritable, may result from environmental insults such as exposure to environmental carcinogens associated with the development of SCC such as cigarette smoke and alcohol. Arguably the most studied epigenetic modification in the context of SCC, hypermethylation of CpG islands, is reported to lead to the downregulation of several miRNA derived from genes in areas of CpG hypermethylation, including miR-34, a miRNA known to play a role in metastasis. Modifications of the DNA packaging proteins, histones, such as methylation and acetylation, are other mechanisms of epigenetic modification which may influence miRNA expression in SCC.

9.4 Functional Consequences of Aberrant MicroRNA Expression in Squamous Cell Carcinoma

miRNA are reported to target a number of genes encoding proteins involved in all key steps of tumourigenesis; carcinogenic transformation, proliferation, invasion and metastasis, as well as contribute to the corruption of cross-talk with the tumour microenvironment which can promote to angiogenesis and immune evasion. miR-124, for example, targets an integrin (ITGB1) with key roles in maintaining tissue architecture in the oral mucosa. Upregulation of miR-124 in HNSCC leads to down-regulation of ITGB1 via two co-operative miR-124-binding sites in the 3′ UTR of the ITGB1 transcript, resulting in decreased adhesion of cells to proteins of the extracellular matrix and increased ability to migrate and invade (Table 9.1).

Table 9.1 miRNA involved in tumour microenvironment remodelling

miRNA	Cell type	Putative targets	Target validation method
miR-9	Cancer cells	CDH1	Luciferase assay
miR-21	Cancer cells, CAFs	PTEN, PDCD4, BTG2, RECK, Smad7	Expression correlation, luciferase assay
miR-26b	CAFs	TNKS1BP1, CPSF7, COL12A1	Expression correlation
miR-29b	Cancer cells	ANGPTL4, LOX, VEGFA, ITGA6	Luciferase assay
miR-31	CAFs	SATB2	Luciferase assay
miR-125b	Cancer cells	HER2, HER3	Luciferase assay
miR-126	Cancer cells	N/A	N/A
miR-126/126*	Tumour tissue containing stromal cells	Sdf-1α	Luciferase assay
miR-143	Cancer cells	MMP-13	Microarray and western blot analyses
miR-145	Cigarette smoke-treated fibroblasts	N/A	N/A
miR-199a	Cancer cells	HER2, HER3	Luciferase assay
miR-223	TAMs	Mef2c	Luciferase assay
miR-320	CAFs	Ets2, Mmp9, Emilin2	Luciferase assay
let-7	Cancer-associated MSCs	IL-6	Luciferase assay

Other miRNA regulate epithelial to mesenchymal transition (EMT), a phenotypic change linked to metastasis in which epithelial cells adopt a migratory mesenchymal form in response to cancer cell and tumour microenvironment-derived factors such as TGF-β1. The miR-200 family of miRNA, amongst others, are reported to modulate EMT by forming a key part of a TGF-β1/ZEB1/SIP1 feedback loop; altered expression of miR-200 family members changes the balance of this homeostatic mechanism and lead to promotion of EMT, thereby conferring metastatic traits on the cell. Other miRNA such as miR-205, miR-21, miR-34a and miR-96 are also reported to play a role in promoting EMT in SCC cells.

A key feature of cancer cells, excessive proliferation, is normally controlled by genes regulating the cell cycle, in response to environmental cues transduced by receptors on the cell surface. As such, altered expression of miRNA able to reduce (or indirectly increase) the expression of any of the genes involved in this process have the capacity to promote proliferation and contribute to tumour growth. In SCC, a number of miRNA elevated in tumours, such as miR-184, have been demonstrated to regulate cancer cell proliferation and promote tumour growth in vivo. In addition, there is widespread evidence for a role for miRNA in blocking programmed cell death (apoptosis) , another key deregulated feature of malignant cells.

9.5 The Role of miRNA in the Tumour Microenvironment

Although the role of miRNA in cancer initiation, growth and metastasis has been intensively studied, the vast majority of these reports have solely examined the contribution of miRNA to the cancer cell phenotype. A relatively small number of studies have interrogated the role of miRNA in reprogramming the tumour microenvironment, but a body of evidence is growing that here too they are key modulators of cell behaviour and communication. The tumour microenvironment of SCCs encompasses the tumour cells, surrounding non-malignant epithelial cells, cancer associated fibroblasts (CAF), extracellular matrix, immune cells, endothelial cells and other less numerous components such as neurites, pericytes and adipocytes, as well as adjacent structures such as bone, depending on the location of the tumour (summarized in Fig. 9.2). Aberrant miRNA expression has been reported in immune cells, endothelial cells and fibroblasts, amongst others. Several studies have identified changes in miRNA expression in CAFs compared to normal fibroblasts.

One miRNA frequently identified as altered in CAF is miR-21, a miRNA with well-described oncogenic effects in cancer cells. In fibroblasts, miR-21 drives fibroblast-to-myofibroblast transdifferentiation and matrix remodelling primarily by repressing two targets; RECK, an inhibitor of MMP activity and Smad7, a negative

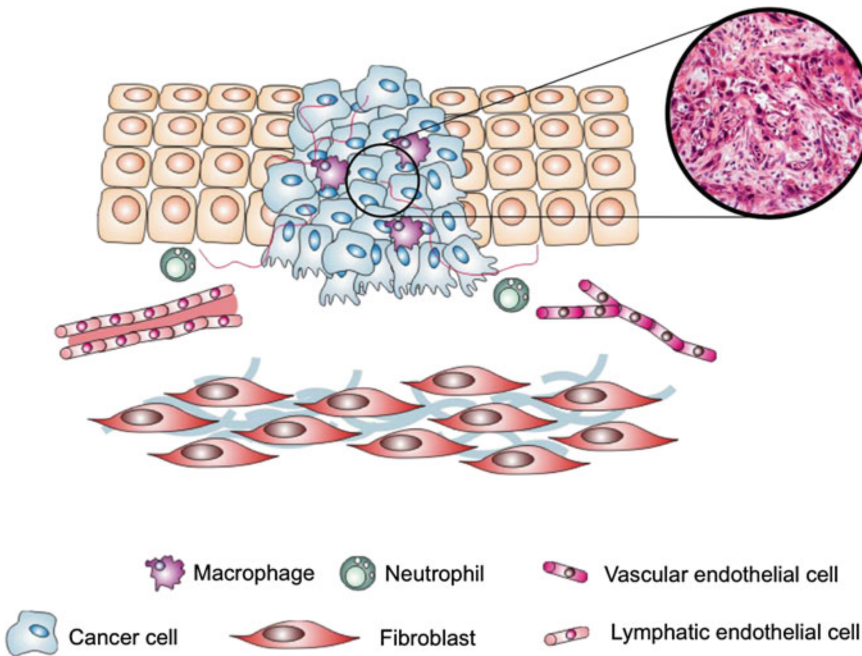

Fig. 9.2 The major components of the SCC tumour microenvironment

regulator of TGFβ signalling. Other examples of miRNAs dysregulated in CAFs are miR-26b, miR-31, and miR-320. Using pathway analysis of differentially expressed proteins, downstream effectors of miR-26 action were identified as glycolysis/TCA cycle and cytoskeletal regulation by Rho GTPases. miR-31, which is down-regulated in CAFs, targets the homeobox gene SATB2, in part responsible for chromatin remodelling and differential expression observed in the CAF phenotype. SAFB2 was shown to increase tumour cell migration and invasion in vivo. miR-320 also plays a key role in tumour microenvironment remodelling. Its expression in tumor stroma is regulated by PTEN (phosphatase and tensin homolog deleted on chromosome 10) and *Pten*-miR-320-*Ets2* axis plays a critical role in promoting tumour angiogenesis and invasion (55). In addition, miR-126/126* is downregulated in metastatic tumour tissue containing stromal cells. This miRNA inhibits the recruitment of MSCs and inflammatory monocytes into the tumour microenvironment by targeting stromal cell-derived factor-1α (SDF-1α) and indirectly suppresses chemokine (C-C motif) ligand 2 (CCL2). miRNA alterations in other stromal cells such as tumour-associated macrophages (TAMs) and cancer-associated MSCs have also been reported.

Some miRNAs dysregulated in cancer cells are also involved in crosstalk between cancer and its microenvironment. miR-9, for instance, promotes angiogenesis which permits cancer cells to disseminate into the circulatory system, while miR-29b modulates MMP activity and TGF-β1 signalling, thereby influencing stromal remodelling. In OSCC, there is a negative correlation between miR-126 levels and tumour progression, angiogenesis, lymphangiogenesis, as well as nodal metastasis. Vascular endothelial growth factor-A (VEGF-A) is proposed to be a target of this miRNA. In addition, miR-29a, miR-29c, and miR-140-3p are up-regulated in metastatic OSCC compared with non-metastatic tissue. Their level of expression is positively correlated with NF-κB which might play a role in tumour microenvironment remodelling. miR-145 is significantly downregulated in oral fibroblasts exposed to cigarette smoke condensate. Furthermore, the expression of miR-145 is inversely correlated with MMP-2 expression, a matrix metalloprotease associated with cancer invasion and metastasis through ECM degradation and remodelling. Fibroblast migration and the ability of fibroblasts to facilitate SCC cell chemotaxis in response to cigarette smoke condensate was inhibited by miR-145 re-expression.

miRNA have also been recently implicated in the establishment of a pro-tumorigenic senescence associated secretory phenotype (SASP) in CAFs in SCC. Senescent CAF are frequently observed in the SCC microenvironment and are postulated to emerge in response to cancer cell-derived oxidative stress as well as chemotherapy and radiotherapy. Kabir et al. (2016) reported miR-335 is upregulated in both senescent normal and cancer associated fibroblasts and promotes secretion of a pro-inflammatory secretome through targeted down-regulation of PTEN. Furthermore, elevated PGE2 and COX-2, a characteristic feature of senescent cells, was shown to drive formation of SASP through the miR-335/PTEN axis.

9.6 miRNA as Signaling Molecules in SCC

In recent years, it has become apparent that extracellular vesicles (EV; frequently referred to as exosomes but actually a larger group of distinct classes of vesicles including microvesicle) play an important role in mediating intercellular communication. SCC cells secrete easily detectable quantities of EVs, including both exosomes and microvesicle, containing a diverse protein and RNA cargo, including miRNA. The miRNA detected in SCC-derived EVs include miR-21, which is reported to be released by hypoxic HNSCC cells and transfer to surrounding normoxic cells. Furthermore, cancer cell derived EVs are able to provoke pro-tumourigenic changes in neighbouring fibroblasts. Cancer cells can also secrete extracellular vesicles that help remodelling their microenvironment such as inducing tube formation in endothelial cells as well as enhancing endothelial cell migration. Interestingly, the release of miRNAs into exosomes is a selective process —which means not every miRNA overexpressed in the cells of origin will be delivered to other cells. For instance, miR-451 and miR-1246 are produced by both malignant and non-malignant epithelial cells but they are selectively secreted in EVs by malignant cells. Significantly, saliva contains high levels of EVs, with miRNA cargo which may be altered in disease.

9.7 Clinical Utility of miRNA as Biomarkers in Squamous Cell Carcinomas

An increasing body of evidence highlights the potential of miRNA as prognostic indicators in SCC, providing a means of relatively non-invasive evaluation of disease progression and monitoring responses to treatment. miR-142-3p, miR-186-5p, miR-195-5p, miR-374b-5p and miR-574-3p were reported to be significantly elevated in plasma from HNSCC patients and this correlated with poor prognosis. Salivary miRNA have also shown promise as potential biomarkers for SCC, with miR-21 and miR-184 levels in whole saliva significantly increased in malignancy and potentially malignant disorders. The miRNA expression pattern in saliva is also reported to distinguish between HPV-positive and—negative HNSCC. A number of studies have also indicated the potential of salivary miRNA as a diagnostic tool in salivary gland tumours.

In recent years, considerable effort has been made to assess the utility of miRNA in EVs as biomarkers in SCC. EV-associated miRNA is very stable, suggesting the capacity to act as a robust biomarker in ‘liquid biopsies’ derived from body fluids such as saliva. Oral swirl samples have recently been shown to be a source of miRNA-containing EVs which have potential as biomarkers, and efforts are ongoing in our laboratory and others to refine procedures for collection and purification of EVs to allow the development of novel salivary biomarkers.

Although miRNA in saliva, whether free (or protein bound) or associated with EVs clearly have potential as biomarkers of SCC development and progression, there remain considerable technical challenges to be overcome. One of these is the inherent difficulties presented by the heterogeneity of saliva, and another is the technical difficulty of normalizing miRNA expression profiles between samples. In the case of EV-associated miRNA, care must be taken to optimize and standardize the method of purification of EV. With these caveats in mind, however, a picture is emerging of salivary miRNA as a useful clinical tool, which holds particular promise for the monitoring progression of premalignant lesions and treatment responses.

9.8 miRNA as Therapeutic Targets—A Novel Treatment Strategy in Squamous Cell Carcinomas?

The in vivo delivery of miRNA inhibitors and mimics holds promise in modulating the effects of miRNA, but several hurdles must be overcome before miRNA-based therapeutics can be pursued in patients. Firstly, the chemical properties of a polyanionic oligonucleotide such as miRNA mean it is unable to pass through hydrophobic cell membranes and is vulnerable to RNAse degradation once localised in the cell. Chemical modification of miRNA-targeting antisense oligonucleotides (ASOs) has shown considerable promise in improving bioavailability and stability, but efficient targeting to specific areas of the body remains challenging. In the oral cavity, topical application may be possible for accessible tumours but this is likely to have limited efficacy and applicability. Nanotechnology based approaches to drug delivery has allowed development of natural and synthetic nanoparticles (1–1000 nm) such as liposomes, exosomes, cyclodextrin, polyethyleneimine (PEI) as well as inorganic carrier molecules (e.g. carbon nanotubes), providing a way of overcoming such physical barriers allowing increase in bioavailability, half-life and reduce immune activation, however the validity of these technologies in the clinical setting is in need of investigation. Several studies have reported positive results from such approaches in vivo [reviewed in Li et al. (2014)], and some miRNA targeting technologies have recently entered early clinical trials (for example MXR34, a mimic of miR-34, in liver cancer).

As yet, to the authors' knowledge, no miRNA-based therapeutics have been trailed in man for SCC, but several have shown promise in preclinical studies in animals.

9.9 Concluding Perspectives

In recent years there has been an explosion in interest and understanding of the role of miRNA in squamous cell carcinomas. Widespread changes in expression of a variety of miRNA have been detected, and some of these have functional significance and/or promise as biomarkers in SCC. In particular, there is considerable interest in the translational potential of miRNA associated with extracellular vesicles in saliva and blood. Much, however, remains to be determined about the prognostic power of salivary miRNA, but clearly these have considerable potential in the clinic for disease monitoring and patient stratification. Considerable advances have been made in developing novel miRNA-directed therapies, raising the prospect of entirely novel treatment approaches in SCC; it should be noted, however, but much work remains to be done to deliver impact on disease outcomes.

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Chapter 10
Envoi—An Appraisal of Targeted Therapies for Head and Neck Cancer

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Abstract Till the end of the last century the standard treatment for recurrent and metastatic squamous cell carcinomas (SCC) of the head and neck was cisplatin/5-fluorouracil. Although polychemotherapy by adding paclitaxel or docetaxel had shown promising progression free survival, toxicities encountered in clinical practice were high. Furthermore, chemo-radioresistance has been identified as an important cause of loco-regional treatment failure following chemotherapy. As an advancement to chemotherapy, strategies targeting tumour growth and proliferation in head and neck cancer have been a focus of intense research over the past two decades. This is following the observations that the growth and metastatic spread of tumours are driven by many genetic aberrations, particularly by overexpression and mutations in EGFR, P13K/mTOR/TP53/RB, Ras/MAPK pathways (Chap. 4). Following these discoveries, recent years have seen the advent of a new generation of agents that directly target these molecules either in malignant cells or cells supporting tumour growth. The most active area of targeted therapy in head and neck cancer in the last decade has been development of monoclonal antibodies (MoAb) targeting tumour antigens on the cell surface or small molecule tyrosine kinase inhibitors (TKI). The majority of inquiry has focussed on EGFR/erbB1/HER1 as EGFR is upregulated in close to 90% of head and neck cancers. Overexpression is also known to be associated with poor prognosis. Trials based on the use of Cetuximab (anti-EGFR) and Trastuzumab [anti-human epidermal growth factor receptor 2 (HER2)] that were developed to block pro-proliferative and anti-apoptotic signalling transmitted by these two transmembrane growth factor receptors are widely reported. Among 181 clinical trials for molecular target therapy on oral/head and neck squamous cell carcinomas registered in ClinicalTrials.gov, 134 studies were based on

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6_10

EGFR inhibitors and 47 concerned other drugs. The therapeutic effects of targeted agents tried in various trials vary with response rates (RRs) between 10 and 40%.

10.1 Introduction

The earlier chapters in this volume have presented available data from studies outlining targeted therapies on specific molecular targets. The aim of this chapter is to make a comprehensive review of the literature regarding on-going clinical trials and published literature on systemic treatment of targeted therapies for head and neck cancer. Two approaches were applied: an appraisal of international trial registries to highlight the agents that are in development for treatment of head and neck squamous cell carcinomas (HNSCC) and we also undertook a systematic review of published trials on targeted therapies on head and neck cancer.

10.2 Information from Trial Registries

The best sources to look out for information on development of new therapies are the international trials registers. A useful application of examining the registered clinical trial data is to shed light on the areas to identify what trials are being conducted, where it is being conducted by whom and how. The revised Declaration of Helsinki, states that "Every clinical trial must be registered in a publicly accessible database before recruitment of the first subject." Worldwide, there is growing number of registries and according to the WIKIPEDIA, 16 registries can be accessed to register a trial: http://en.wikipedia.org/wiki/Clinical_trials_registry.

A study in 2013 (Clinical trials registry. Wikipedia) identified the following top five registries that list most trials undertaken globally. The enrolled number of trials in these registries at the time is shown in parenthesis. Many of the trials from Asia are also listed in the ClinicalTrials.gov.

1.	ClinicalTrials.gov	(150,551)
2.	EU register	(21,060)
3.	Japan Registries Network (JPRN)	(12,728)
4.	ISRCTN	(11,794)
5.	Australia and New Zealand (ANZCTR)	(8,216)

We searched for randomized clinical trials that listed targeted therapies for head and neck cancer in these five major trial registries.

10.3 Systematic Review

Electronic searches including MEDLINE, PubMed and trial registries and manual searches of references in published studies or abstracts published in proceedings of meetings were used to identify the relevant literature. Search terms included squamous cell carcinomas of head and neck, clinical trial, metastatic or advanced cancers. The articles were selected for inclusion if they met the following criteria: randomized controlled trials (RCTs) with appropriate control groups. Some single arm trials were included if there was no evidence from phase III clinical trials.

10.3.1 Inclusion Criteria

We prioritized for randomized trials. To select studies for inclusion in our analysis, we checked for the study status entered in the trial registries, and included only the trials that were completed or currently recruiting studies. For some novel drugs, active, but not recruiting or of other status were also included.

In the case of Cetuximab, because of more than 160 studies were found, we only included completed, and trials with results or published studies.

10.3.2 Exclusion Criteria

Active but not recruiting (without results), not yet recruiting, suspended, withdrawn trials or of unknown status were excluded from the summary results. Enrolled trials with few case numbers were excluded, except in rare studies.

10.3.3 Assessments

We transcribed the selected trials along with their stated primary outcome measures. The primary endpoint in phase III cancer clinical trials is often survival. However, trials in early disease, for which the majority of patients are expected to remain alive for many years, may use recurrence or relapse-free survival as the primary endpoint. Quality of life and healthcare economics are secondary endpoints within phase III trials.

The disease outcome measures (or patient survival) related to the interventions for head and neck cancer have been rated by several different measures: Overall survival (OS), Disease free survival (DFS), Event-free survival (EFS), Progression free survival (PFS), Rate of disease control (RDC), Time to progression (TTP), Patients free of progression (PFR), Failure free survival (FFS) and Local regional

control (LRC), Completion rate (CR), Pathology (P), Pharmacokinetics (PK), Overall response rate (ORR), Tumor resolution (TR) and compliance (C). Some studies (in Phases I and II) also measured Toxicity/Safety (T/S), Adverse events (AE), and Maximum tolerated dose (MTD).

Tables 10.1 and 10.2 list the outcome from our searches of Trial Registers by the NCT numbers. Table 10.1 lists all individual trials on anti EGFR therapies and in Table 10.2 other agents that have been used in human studies. Among 181 clinical trials (Tables 10.1 and 10.2) related to molecular target therapy on oral/head and neck squamous cell carcinoma registered in ClinicalTrials.gov, 134 studies were based on EGFR inhibitors and 47 concerned other drugs.

The EGFR inhibitors utilized in trials include EGFR MoAb (47%), EGFR TKI (29%), dual TKI (15%) and MKI (9%). EGFR MoAb used in trials include cetuximab (27%), bevacizumab (6%), panitumumab (5%), nimotuzumab (4%), zalutumumab (4%), RO5083945 (imgatuzumab) (1%) and Sym004 (anti-EGFR antibody mixture) (1%). The EGFR TKI included gefitinib (44%), erlotinib (12%), pazopanib (3%), nilotinib (2%), pazopanib (2%), MGCD265 (glesatinib) (2%), axitinib (VEGFR-TKI) (2%) and dual TKI (34%) (afatinib, axitinib for EGFR/HER2 and MEHD7945A for EGFR/HER3). MKI included vandetanib (VEGFR inhibitor) (56%), sorafenib (33%), sunitinib (33%) and MM-151 (anti-EGFR antibody mixture) (11%).

Other molecular target drugs entered into trials were mTOR inhibitors; everolimus, P13K inhibitor (BYL719/BKM120), temsirolimus, rapamycin and ridaforolimus, MET inhibitor (LY2801653 and foretinib), adenovirus gene therapy (INGN 201), IGF-1R inhibitor (cixutumumab), PARP-1 inhibitor (olaparib), AKT inhibitor (MK2206), ALK1 inhibitor (dalantercept), AMPK activator (metformin), Aurora A kinase inhibitor (alisertib) , Bcr-Abl TKI (dasatinib) , CDK inhibitor (P276-00) , Hedgehog inhibitor (saridegib), Hypoxic radiosensitizer (nimorazole), Immunomodulatory drugs (IMiDs) (lenalidomide), MEK inhibitor (tarametinib), PDK inhibitor (dichloroacetate), proteasome inhibitor (bortezonib), RTK inhibitor (MGCD516), Src/Abl kinase inhibitor (saracatinib) and toll-like receptor (TLR) 8 agonist (VTX-2337) .

Most clinical trials on targeted therapies on head and neck cancer have been conducted in USA (127; 70%) followed by EU (47; 26%), Asia (21; 12%), East Europe and Russia (9; 5%), Australia (4; 2%), South America (4; 2%), Canada (3; 2%), Mexico (2; 1%), Israel (1; 1%) and South Africa (1; 1%).

The status of the studies registered are as follows: completed (57%), recruiting (35%), active, not recruiting or active, not recruiting, have results (3%), on going but not recruiting (2%) terminated (2%) and enrolling by invitation (1%).

Study phases are listed as: Phase I (20%), Phase II (53%), Phase I/II (14%), Phase III (12%) and combined Phase II/III (1%). Study designs are stated as, single (55%), randomized (41%) and non-randomized (4%). The number of patients entered to each trial ranged from 3 to 710 (median: 54). Among the 63 randomized trials, 31 studies (49%) have been completed or terminated, and among those, 17 (55%) studies have been published.

Table 10.1 Summary of clinical trials for molecular target therapy on oral/head and neck squamous cell carcinoma (EGFR inhibitors)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	USA	NCT00004227	Terminated	III	Recurrent or metastatic SCCHN	**Arm1**: RT **Arm2**: RT Cetuximab (Randomized)	32	LRC	N/A	Bonner et al. (2006)
EGFR TKI	Gefitinib	USA	NCT00015964	Completed	II		**Arm**: ZD1839 (Single arm)	51	LRC	N/A	–
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00023959	Completed	I	Recurrent, previously radiated or poor-prognosis, treatment-naive HNC	**Arm**: Bevacizumab, Hydroxyurea, Fluorouracil, RT (Single arm)	39	MTD	N/A	Seiwert et al. (2013)
EGFR TKI	Gefitinib	USA	NCT00024089	Completed	II		**Arm**: Gefitinib (Single arm)	60	LRC	N/A	–
EGFR TKI	Gefitinib	USA	NCT00033449	Completed	I	Untreated locally advanced HNSCC	**Arm**: Gefitinib, RT with/without Cisplatin (Single arm)	30	T/S	N/A	Chen et al. (2007)
EGFR TKI	Erlotinib	USA	NCT00055770	Completed	I,II	Recurrent or metastatic or radiation and SCCHN	**Arm**: Erlotinib, Docetaxel (Single arm)	45	MTD, LRC	N/A	Kraut et al. (2011)
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00055913	Completed	I,II	Recurrent or metastatic SCCHN	**Arm1**: Bevacizumab, Erlotinib **Arm2**: Bevacizumab, Erlotinib (Randomized)	58	MTD, LRC	N/A	Cohen et al. (2009)
EGFR TKI	Gefitinib	USA	NCT00083057	Completed	I	Untreated stage III, IVA or IVB HNSCC	**Arm**: Gefitinib, Paclitaxel, RT (Single arm)	30	T/S, MTD	N/A	Van Waes et al. (2010)
EGFR TKI	Gefitinib	USA	NCT00088907	Completed	III	Incurable recurrent metastatic SCCHN	**Arm 1**: Docetaxel, Placebo **Arm2**: Docetaxel, Gefitinib (Randomized)	330	OS	OS: 5.98 (4.93–7.43) in Arm1 and 7.33 (5.75–8.44) in Arm2 (P = .60)	Argiris et al. (2013b)

(continued)

Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	USA	NCT00089297	Completed	II	Resectable HNSCC (stage III or IV)	**Arm:** Cetuximab, Paclitaxel, Carboplatin, RT (Non-Randomized)	74	EFS	EFS at 1 Year: 0.79 (95% CI; 0.69–0.89)	Wanebo et al. (2014)
EGFR MoAb	Zalutumumab	Denmark and Sweden	NCT00093041	Completed	I,II	No currable or palliative	**Arm**: Zalutumumab (Non-Randomized)	28	AE	All but one adverse event	Bastholt et al. (2007)
EGFR MoAb	Cetuximab	USA	NCT00096174	Ongoing but not recruiting	II	Locally advanced HNSCC	**Arm**: Cetuximab, Cisplatin, RT (Single arm)	69	PFS	PFS 2-year: 47% (95%CI; 33–61%)	Egloff et al. (2014)
MKI	Sorafenib	USA	NCT00096512	Completed	II		**Arm**: Sorafenib (Single arm)	40	LRC, PFS, OS, T/S	N/A	–
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT00098631	Completed	II	Recurrent/metastatic SCCHN	**Arm**: Lapatinib (with/without prior EGFR inhibitor exposure) (Single arm)	88	LRC, PFS	N/A	de Souza et al. (2012)
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00101348	Completed	I,II		**Arm1**: Erlotinib Hydrochloride, Cetuximab, Bevacizumab **Arm2**: Erlotinib Hydrochloride, Cetuximab (Randomized)	66	MTD	N/A	–
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT00114283	Completed	II		**Arm**: Lapatinib (Single arm)	30	LRC	N/A	–
EGFR MoAb	Cetuximab	EU and others	NCT00122460	Completed	III	Unresected, locally advanced SCCHN	**Arm1**: Cetuximab, Platinum (Cisplatin or Carboplatin), 5Fluorouracil (5-FU) **Arm2**: Platinum (Cisplatin or Carboplatin), 5Fluorouracil (Randomized)	442	OS	OS: 10.1 (95%CI; 8.6–11.2) in Arm1 and 7.4 (95%CI; 6.4–8.3) in Arm2 (p = 0.036)	Vermorken et al. (2008)
EGFR MoAb / VEGFR	Bevacizumab (Avastin) Erlotinib	USA	NCT00140556	Completed	0	Previously untreated locally advanced SCCHN	**Arm**: RT, Cisplatin, Bevacizumab (Avastin), Erlotinib (Single arm)	28	TR	TR: 25 out of 26 participants	Yoo et al. (2012)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR TKI	Gefitinib	France	NCT00169221	Completed	II	Planned surgery for locally advanced SCCHN	**Arm1**: RT, Cisplatin **Arm2**: Gefitinib, RT, Cisplatin (Randomized)	54	DFS	N/A	Thariat et al. (2012)
EGFR TKI	Gefitinib (ZD-1839)	USA	NCT00185835	Completed	I		**Arm**: ZD-1839,Cisplatin (Randomized)	10	T/S	N/A	–
EGFR TKI	Gefitinib	USA	NCT00193284	Completed	II	Untreated locally advanced HNSCC	**Arm**: Gefitinib, Docetaxel, Carboplatin, Fluorouracil, RT (Single arm)	50	ORR	N/A	Hainsworth et al. (2009)
EGFR TKI	Gefitinib	USA	NCT00195078	Completed	I,II		**Arm**: ZD1839 (IRESSA), Cisplatin, RT (Single arm)	29	LRC	N/A	–
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00203905	Completed	II		**Arm1**: 5-Fluorouracil, Hydroxyurea **Arm2**: 5-Fluorouracil, Hydroxyurea, Bevacizumab (Randomized)	23	PFS	N/A	–
EGFR TKI	Gefitinib	USA, EU and others	NCT00206219	Completed	III		**Arm1**: Gefitinib **Arm2**: Methotrexate (Randomized)	477	OS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT00226239	Completed	II		**Arm**: Docetaxel, Cisplatin, Cetuximab, RT (Single arm)	39	LRC	N/A	Argiris et al. (2010)
EGFR TKI	Gefitinib	Hong Kong and Singapore	NCT00228488	Completed	II		**Arm**: Iressa, RT (Single arm)	60	P	N/A	–
EGFR TKI	Gefitinib	USA, EU and others	NCT00229723	Completed	II	Previously untreated, unresected, stage III/IV non-metastatic SCCHN	**Arm1**: RT, Cisplatin, Placebo **Arm2-3,6–7**: Gefitinib, Cisplatin, RT, Placebo **Arm4–5**: Gefitinib, Cisplatin, RT (Randomized)	224	LRC	LRC at 2 years: Arm1 to Arm7: 21/60, 7/24, 15/31, 7/31, 7/24, 9/34, 9/22 participants respectively	Gregoire et al. (2011)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR TKI	Gefitinib	Italy	NCT00233636	Completed	II		**Arm**: Gefitinib, RT (Single arm)	28	LRC	N/A	–
EGFR TKI	Gefitinib	Finland	NCT00239304	Completed	I,II		**Arm**: Gefitinib, Cisplatin, RT (Single arm)	40	T/S	N/A	–
EGFR TKI	Gefitinib	Spain	NCT00242749	Completed	II		**Arm**: Gefitinib, RT, Cisplatin (Single arm)	47	DFS	N/A	–
EGFR TKI	Gefitinib	Spain	NCT00242762	Completed	II		**Arm**: ZD1839, Docetaxel, Cisplatin (Single arm)	36	T/S	N/A	–
EGFR TKI	Gefitinib	UK	NCT00255476	Completed	II		**Arm**: Gefitinib, Cisplatin, 5-flourouracil (Randomized) Study arm: not provided	64	LRC	N/A	–
EGFR MoAb	Cetuximab	USA and Canada	NCT00265941	Ongoing but not recruiting	III		**Arm1**: Cisplatin, RT **Arm2:** Cisplatin, Cetuximab, RT (Randomized)	720	DFS	N/A	Ang et al. (2014)
EGFR MoAb	Cetuximab	USA	NCT00301028	Completed	II	Locally advanced HNSCC	**Arm**: Cetuximab, Carboplatin, Paclitaxel, Surg, RT (Single arm)	48	LRC	CR: 83%	Kies et al. (2010)
EGFR MoAb	Erbitux (Cetuximab)	USA	NCT00343083	Completed	II	Locally advanced HNSCC	**Arm**: Erbitux, Paclitaxel & Carboplatin, RT (Single arm)	43	LRC	3-year LRC: 72%	Suntharalingam et al. (2012)
EGFR TKI	Gefitinib	USA	NCT00352105	Completed	I,II	Previously untreated stage III, IVA, or IVB SCCHN	**Arm**: Cisplatin, Fluorouracil, Iressa RT (Non-Randomized)	60	OS	2- and 3-year OS: 80% and 71%	Rodriguez et al. (2012)
Dual TKI (EGFR/HER2)	Lapatinib	France, Greece, India, Peru and Spain	NCT00371566	Completed	II	Newly diagnosed stage III/IVA/IVB SCCHN	**Arm1**: Placebo **Arm2**: Lapatinib (Randomized)	107	P	Change from baseline of the apoptotic index during treatment phase: 6.2 ± 12.10% in Arm:1 and 4.2 ± 5.53% in Arm2	Del Campo et al. (2011)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Zalutumumab	UK, Belgium, France, Hungary and others	NCT00382031	Completed	III	Incurable recurrent or metastatic	**Arm1**: Zalutumumab in combination with Best Supportive Care **Arm2**: Best Supportive Care (Randomized)	286	OS	Median OS: 6.7 months (95% CI; 5.8–7.0) in the Arm 1 and 5.2 months (4.1–6.4) in the Arm 2	Machiels et al. (2011)
MKI	Sunitinib	USA	NCT00387335	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Sunitinib (Single arm)	22	LRC	Tumor response rate: 6.7 (95%CI; 0.2–31.9) in Arm1 and 0 (95%CI; 0–41.0) in Arm2	Choong et al. (2010)
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00392704	Completed	II	Previously untreated locally advanced SCCHN	**Arm**: Bevacizumab, Erlotinib, Paclitaxel, 5-FU, RT (Single arm)	60	PFS	2-year PFS probability: 83%	Haisworth et al. (2011)
EGFR MoAb	Zalutumumab	USA, Belgium, France, Netherlands and Sweden	NCT00401401	Completed	I,II		**Arm**: Zalutumumab, Cisplatin, Procedure, RT (Single arm)	30	MTD	Adverse event: 100%	–
MKI	Sunitinib	Belgium and France	NCT00408252	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Sunitinib (Single arm)	54	LRC	N/A	Machiels et al. (2010)
EGFR MoAb / VEGFR	Bevacizumab	USA	NCT00409565	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Cetuximab, Bevacizumab (Single arm)	48	ORR	The ORR: 16% (95%CI; 7–24%) and the disease control rate (DCR): 73%	Argiris et al. (2013a)
EGFR TKI	Erlotinib	USA	NCT00410826	Completed	II	Locally advanced HNSCC	**Arm1**: Cisplatin, RT **Arm2**: Erlotinib, Cisplatin, RT (Randomized)	204	LRC	CR: 40% in Arm1 and 51% in Arm2	Martins et al. (2013)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR TKI	Erlotinib	Spain	NCT00412217	Completed	III		**Arm1**: Erlotinib **Arm2:** Standard care of treatment (Randomized)	94	FFS	N/A	–
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT00424255	Completed	III	Resected stage II to IVA SCCHN	**Arm1**: Lapatinib, Cisplatin, RT **Arm2**: Cisplatin, Placebo, RT (Randomized)	688	DFS	Median DFS: 53.6 months (95%CI 45.8-NA) in Arm1 and NA (95%CI 54.6-NA) in Arm2 (p = 0.2251)	Harrington et al. (2015)
MKI (VEGFR inhibitor)	Vandetanib	USA	NCT00450138	Completed	I	Unresected SCCHN (stage III–IV with no proven haematogenous metastatic disease)	**Arm1:** ZD6474 (Vandetanib), RT **Arm2:** ZD6474 (Vandetanib), Cisplatin, RT (Non-randomized)	33	T/S	N/A	Papadimitrakopoulou et al. (2014)
EGFR MoAb	Panitumumab	USA	NCT00454779	Completed	II		**Arm 1**: Panitumumab, Docetaxel, Cisplatin **Arm2**: Docetaxel, Cisplatin (Randomized)	113	PFS	PFS: 6.9 months (95%CI; 4.7–8.3) in the Arm 1 and 5.5 (95%CI; 4.1–6.8) in the Arm 2. HR: 0.629, 95%CI (0.395–1.002) (p = 0.051)	–
MKI (VEGFR inhibitor)	Vandetanib	USA	NCT00459043	Completed	II	Recurrent or metastatic SCCHN (not amenable to primary surgical resection or radiotherapy)	**Arm1**: Docetaxel, **Arm2**: Docetaxel, ZD6474 (Randomized)	30	LRC	PR: 7% (95%CI; 0.2–33.8) in Arm1 and 13% (95%CI; 1.6–40.4) in Arm2	Limaye et al. (2013)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Panitumumab	Belgium and others	NCT00460265	Completed	III	Recurrent or metastatic SCCHN	**Arm1**: Panitumumab, Cisplatin, 5-FU **Arm2**: Cisplatin and 5-FU (Randomized)	658	OS	Median OS: 11.1 months (95%CI; 9.8–12.2) in the Arm 1 and 9·0 months (95%CI; 8.1–11.2) in the Arm 2 (HR: 0.873, 95%CI; 0.729–1.046; $p = 0.1403$)	Vermorken et al. (2013a)
EGFR MoAb	Panitumumab	USA	NCT00500760	Completed	II	Locally advanced HNSCC	**Arm1**: Panitumumab, RT, Chemo, RT **Arm2**: RT, Cisplatin (Randomized)	153	LRC	2 years LRC: 0.61 (95%CI; 0.50–0.71) in the Arm1 and 0.68 (95%CI; 0.54–0.78) in the Arm 2	Mesía et al. (2015)
Dual TKI (EGFR/HER2)	Afatinib / Cetuximab	Belgium, France, Spain and USA	NCT00514943	Completed	II		**Arm1**: Afatinib **Arm2**: Cetuximab (Randomized)	124	Tumor shrinkage before crossover	Afatinib: −3.86 mm (3.62) Mean (Standard Error) Cetuximab: −2.37 mm (3.47) ($p = 0.761$)	–
EGFR TKI	Gefitinib	USA	NCT00519077	Completed	II	Refractory currable recurrent / metastatic SCCHN	**Arm**: Gefitinib (Single arm)	44	LRC	Response (PR): 6.81%	Perez et al. (2012)
EGFR MoAb	Cetuximab	USA and Canada	NCT00524017	Completed	II		**Arm1**: Cetuximab **Arm2**: Follow-up (Randomized)	35	P	N/A	–

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Zalutumumab	USA	NCT00542308	Completed	II	Platinum-refractory SCCHN	**Arm**: Zalutumumab (Single arm)	90	OS	Median OS: 5.3 months (95%CI; 4.1–7.1)	Saloura et al. (2014)
EGFR MoAb	Panitumumab	USA	NCT00547157	Completed	II	Unresected, locally advanced SCCHN	**Arm1**: RT, Panitumumab **Arm2**: RT, Cisplatin (Randomized)	152	LRC	2 years LRC: 51% (95%CI; 40–62) in the Arm1 and 61% (95%CI; 47–72) in the Arm 2	Giralt et al. (2015)
EGFR MoAb/ VEGFR	Bevacizumab	USA	NCT00588770	Recruiting	III		**Arm1**: Docetaxel, Cisplatin **Arm2**: Docetaxel, Cisplatin, Bevacizumab **Arm2A**: Docetaxel, Carboplatin **Arm 2B**: Docetaxel, Carboplatin, Bevacizumab **Arm 3A**: Cisplatin, Fluorouracil **Arm 3B:** Cisplatin, Fluorouracil, Bevacizumab **Arm 4A:** Carboplatin, Fluorouracil **Arm 4B:** Carboplatin, Fluorouracil, Bevacizumab (Randomized)	400	OS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT00660218	Recruiting	I,II		**Arm**: Paclitaxel, Cetuximab, RT (Single arm)	60	MTD, LRC	N/A	–
EGFR TKI	Gefitinib	Switzerland	NCT00681967	Completed	I		**Arm**: Gefitinib (Single arm)	30	T/S	N/A	–
EGFR TKI	MGCD265 (Glesatinib)	USA	NCT00697632	Recruiting	I		**Arm**: MGCD265 (Single arm)	150	T/S	N/A	–

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	EU	NCT00705016	Completed	I,II	Recurrent or metastatic SCCHN	**Arm1**: Cilengitide (once), Cetuximab, 5-FU, Cisplatin **Arm2**: Cilengitide (twice), Cetuximab, 5-FU, Cisplatin **Arm3**: Cetuximab, 5-FU, Cisplatin (Randomized)	184	PFS	PFS: 6.4 months (95%CI; 5.4–8.7) in Arm1, 5.6 months (95%CI; 4.0–6.1) in Arm2 and 5.7 months (95%CI; 4.2–9.5) in Arm3	Vermorken et al. (2013b)
MKI (VEGFR inhibitor)	Vandetanib	USA	NCT00720083	Completed	II		**Arm1**: ZD6474 (vandetanib), Cisplatin, RT **Arm2**: Cisplatin, RT (Randomized)	34	DFS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT00736944	Active, not recruiting, has results	II	Locally advanced HNSCC	**Arm**: Abraxane, Cetuximab, Cisplatin, 5-FU, RT (Single arm)	30	LRC	Clinical CR rate at the primary tumor: 53%	Adkins et al. (2013)
EGFR MoAb	Panitumumab	USA	NCT00756444	Completed	II		**Arm 1**: Panitumumab, Cisplatin, 5-FU **Arm 2**: Cisplatin, 5-FU (Randomized)	67	PK	AUC of total plasma cisplatin-derived platinum levels: 58,000 ± 9,530 [ng hr/mL] in Arm1 and 62,300 ± 10,200 in Arm2	–
EGFR TKI	Erlotinib	USA	NCT00779389	Completed	I		**Arm1:** Erlotinib **Arm2:** Dasatinib + Placebo **Arm3:** Erlotinib plus Dasatinib **Arm4:** Placebo (Non-randomized)	58	P	N/A	–

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Panitumumab	USA	NCT00798655	Recruiting	II		**Arm**: Panitumumab, Cisplatin plus RT (Single arm)	46	PFS	N/A	–
MKI	Sorafenib	USA	NCT00815295	Completed	I,II		**Arm**: Sorafenib, Cetuximab (Single arm)	31	OS	N/A	–
EGFR TKI	Gefitinib	Belgium and Spain	NCT00820417	Completed	I		**Arm**: Cetuximab, Gefitinib (Single arm)	63	MTD	N/A	–
EGFR MoAb	Cetuximab	Japan	NCT00865098	Completed	II	Untreated locally advanced HNSCC	**Arm**: Cetuximab, RT (Single arm)	27	CR	Completion rate: 100% (95%CI; 84.6–100.0)	Okano et al. (2013)
EGFR MoAb	Cetuximab	USA	NCT00904345	Recruiting	II		**Arm**: Cetuximab, RT (Single arm)	50	P	N/A	–
MKI	Sunitinib	USA	NCT00906360	Completed	I		**Arm**: Sunitinib, RT, Cetuximab (Single arm)	36	MTD	N/A	–
EGFR MoAb	Nimotuzumab	China	NCT00910117	Completed	II		**Arm**: Nimotuzumab, Cisplatin, 5-FU (Single arm)	40	LRC	N/A	–
EGFR MoAb	Nimotuzumab	Singapore	NCT00957086	Recruiting	III		**Arm1**: Nimotuzumab, Cisplatin, RT **Arm2**: Cisplatin, RT (Randomized)	710	DFS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT00957853	Recruiting	II		**Arm1**: Cetuximab, Surg **Arm2**: IMC-A12, Surg **Arm3**: Cetuximab, IMC-A12, Surg (Randomized)	60	P	N/A	–
EGFR MoAb	Cetuximab	Japan	NCT00971932	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Cetuximab, Cisplatin/Carboplatin, 5-FU (Single arm)	33	LRC	Best overall response (BOR) : 36.4% (95%CI; 20.4–54.9)	Yoshino et al. (2013)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR TKI	Pazopanib	USA	NCT01012362	Completed	I		**Arm**: Pazopanib, Lxabepilone (Single arm)	31	T/S	N/A	–
EGFR TKI	Erlotinib	USA	NCT01013831	Terminated	I		**Arm**: Erlotinib (Single arm)	60	P	N/A	–
EGFR MoAb	Cetuximab	USA	NCT01040832	Completed	II	Recurrent or metastatic SCCHN	**Arm1**: Cetuximab, EMD 1201081 **Arm2**: Cetuximab (Randomized)	107	PFS	PFS: 1.5 months (95%CI; 1.3–2.6) in Arm1 and 1.9 months (95%CI; 1.5–2.9) in Arm2	Ruzsa et al. (2014)
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT01044433	Completed	II		**Arm**: Lapatinib, Capecitabine (Single arm)	44	OS	N/A	–
EGFR MoAb	RO5083945 (Imgatuzumab)	France, Italy, Netherlands, Spain and United Kingdom	NCT01046266	Completed	I		**Arm1**: RO5083945 **Arm2**: cetuximab (Randomized)	62	P	N/A	–
EGFR MoAb	Zalutumumab	UK, Belgium, France, Hungary and Slovakia	NCT01054625	Completed	I,II		**Arm1**: Zalutumumab (4 mg/kg) **Arm 2**: Zalutumumab (8 mg/kg) **Arm3**: Zalutumumab (16 mg/kg) (Non-Randomized)	31	PK	N/A	–
EGFR MoAb	Cetuximab	USA	NCT01057589	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Cetuximab, Pemetrexed, Carboplatin or Cisplatin, Folic Acid Dietary, Vitamin B12 (Single arm)	66	PFS	PFS: 4.4 months (95%CI; 3.6–5.4)	Vermorken et al. (2013b)
EGFR TKI	Erlotinib	USA	NCT01064479	Recruiting	II		**Arm1**: Docetaxel, Cisplatin or Carboplatin, Erlotinib **Arm2:** Cisplatin or Carboplatin, Placebo (Randomized)	120	PFS	N/A	–

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	USA,Canada and Mexico	NCT01081041	Active, not recruiting, has results	II	Untreated recurrent and/or metastatic SCCHN	**Arm1,2**: Cetuximab, Cisplatin, Carboplatin, 5-FU (Randomized)	187	T/S	AEs with the highest incidence: nausea, fatigue, and hypomagnesemia in both arms. No significant difference between two Arms	Soulières et al. (2016)
EGFR MoAb	Cetuximab	USA	NCT01087970	Completed	II		**Arm**: Cetuximab, Pemetrexed, Carboplatin or Cisplatin (Single arm)	69	PFS	PFS: 5.1 months (95%CI; 3.9–6.0)	–
EGFR MoAb	Cetuximab	China	NCT01177956	Completed	III	Recurrent or metastatic SCCHN	**Arm**: Cetuximab, Cisplatin, 5-FU (Single arm)	73	LRC	Best overall response (BOR) until cut-off date: 54.4% (95%CI; 41.9–66.5)	Guo et al. (2015)
EGFR TKI	Gefitinib	USA	NCT01185158	Completed	II		**Arm**: ZD1839 (Single arm)	46	To assess the activity	N/A	–
EGFR TKI	Gefitinib	USA	NCT01185171	Completed	II		**Arm**: ZD 1839 (Single arm)	65	LRC	N/A	–
EGFR MoAb	Cetuximab	Italy	NCT01216020	Recruiting	II		**Arm1:** RT, Cisplatin **Arm2:** RT, Cetuximab (Randomized)	140	C	N/A	Magrini et al. (2016)
EGFR MoAb	Cetuximab	USA and Canada	NCT01302834	Ongoing but not recruiting	III		**Arm1**: Cisplatin, RT **Arm2:** Cetuximab, RT (Randomized)	706	OS	N/A	–

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Panitumumab	USA	NCT01305772	Completed	II		**Arm1**: Panitumumab, Surg, RT **Arm2**: Panitumumab, RT (Non-Randomized)	6	LRC	N/A	–
EGFR MoAb	Cetuximab, Erlotinib	USA	NCT01316757	Recruiting	II		**Arm**: Cetuximab, Paclitaxel, Carboplatin, Erlotinib (Single arm)	43	LRC	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	Multi-centre	NCT01345669	Recruiting	III		**Arm1**: Afatinib **Arm2**: Placebo (Randomized)	669	DFS	N/A	–
EGFR MoAb	Nimotuzumab	China	NCT01393184	Enrolling by invitation	II		**Arm1**: Nimotuzumab, RT **Arm2**: RT (Randomized)	92	LRC	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	Multi-centre	NCT01345682	On going but not recruiting	III	No currable recurrent and/or metastatic SCCHN	**Arm1**: Afatinib **Arm2**: Methotrexate (Randomized)	483	PFS	Median PFS in Afatinib: 2.63 months (95%CI; 2.00–2.73) Methotrexate: 1.74 months (95% CI; 1.48–2.40) (p = 0.0296)	Machiels et al. (2015a)
EGFR TKI	Gefitinib	UK	NCT01405846	Recruiting	II		**Arm**: Gefitinib (Single arm)	40	P	N/A	–
MKI (VEGFR inhibitor)	Vandetanib	USA	NCT01414426	Recruiting	II		**Arm1**: Vandetanib **Arm2**: Placebo (Randomized)	54	P	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	France	NCT01415674	Recruiting	II		**Arm 1**: Afatinib **Arm2**: No intervention (Randomized)	60	P	N/A	–
EGFR MoAb	Sym004 (anti-EGFR antibody mixture)	Belgium, France and Germany	NCT01417936	Completed	II	Recurrent and/or metastatic SCCHN with acquired resistance to anti-EGFR monoclonal antibody	**Arm**: Sym004 (Single arm)	26	PFS	N/A	Machiels et al. (2015b)

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Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Nimotuzumab	China	NCT01425736	Completed	II		**Arm1**: Nimotuzumab, Docetaxel, Cisplatin, Fluorouracil **Arm2**: Docetaxel, Cisplatin, Fluorouracil (Randomized)	91	RDC	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	France	NCT01427478	Recruiting	III		**Arm1**: Afatinib **Arm2**: Placebo (Randomized)	315	DFS	N/A	-
EGFR MoAb	Erbitux (Cetuximab)	China	NCT01434394	Recruiting	II,III		**Arm1**: Erbitux, Docetaxel, Cisplatin, Surg, RT **Arm2**: Surg, RT (Randomized)	243	P	N/A	–
EGFR MoAb	Cetuximab	Switzerland	NCT01435252	Recruiting	II		**Arm1**: Cetuximab (concurrent) **Arm2**: Cetuximab (concurrent and consolidation) (Randomized)	60	LRC	N/A	–
EGFR TKI	Gefitinib	Korea	NCT01449201	Recruiting	II		**Arm**: PF-00299804 (Single arm)	49	LRC	N/A	–
EGFR MoAb	Cetuximab	USA	NCT01468896	Completed	I,II		**Arm**: Cetuximab, Recombinant Interleukin-12 (Single arm)	47	T/S	N/A	–
VEGFR TKI	Axitinib	USA	NCT01469546	Recruiting	II		**Arm**: Axitinib (AG-013736) (Single arm)	40	PFS	N/A	–
EGFR TKI	Erlotinib	USA	NCT01488318	Completed	II	Previously untreated HNSCC (Stage II-IVA)	**Arm1**: Erlotinib 150 mg **Arm2**: Erlotinib (150mg) plus sulindac **Arm3**: Placebo (Randomized)	47	P	Ki67 was significantly decreased by erlotinib or erlotinib-sulindac ($p = 0.04$)	Gross et al. (2014)
EGFR MoAb	Nimotuzumab	China	NCT01516996	Recruiting	II		**Arm1**: Docetaxel, Cisplatin, RT, Nimotuzumab **Arm2**: Docetaxel, Cisplatin, RT (Randomized)	80	LRC	N/A	–

(continued)

Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
MKI	MM-151 (anti-EGFR antibody mixture)	USA	NCT01520389	Recruiting	I		**Arm**: MM-151 or MM-151, Irinotecan (Non-Randomized)	115	MTD	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	Belgium and Italy	NCT01538381	Recruiting	II		**Arm1**: Afatinib **Arm2**: Observation (Randomized)	30	LRC	N/A	–
EGFR MoAb	Cetuximab	USA	NCT01566435	Active, not recruiting, has results	II		**Arm**: Paclitaxel, Cisplatin, 5-FU, Cetuximab (Single arm)	30	LRC	Complete Response (CR): 76.7%	–
Dual TKI (EGFR/HER3)	MEHD7945A (Duligotuzumab)	USA, EU and others	NCT01577173	Recruiting	II		**Arm1**: MEHD7945A **Arm2**: Cetuximab (Randomized)	110	PFS	N/A	–
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT01612351	Recruiting	II		**Arm**: Carboplatin, Paclitaxel, Lapatinib, Cisplatin,RT (Single arm)	40	LRC	N/A	–
EGFR MoAb	Cetuximab	USA	NCT01637194	Completed	I		**Arm**: Cetuximab, Everolimus (Single arm)	12	T/S	N/A	–
Dual TKI (EGFR/HER2)	Lapatinib	USA	NCT01711658	Recruiting	II		**Arm1**: RT, Cisplatin, Lapatinib **Arm2**: RT, Cisplatin, Lapatinib (Randomized)	176	OS	N/A	–
EGFR TKI	Pazopanib	USA	NCT01716416	Recruiting	I		**Arm**: Pazopanib, Cetuximab (Single arm)	33	MTD	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	USA	NCT01732640	Recruiting	I,II		**Arm**: Afatinib, Paclitaxel, Carboplatin, Cisplatin, RT (Single arm)	71	MTD, LRC	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	USA	NCT01783587	Recruiting	I		**Arm**: Afatinib, Docetaxel, RT (Single arm)	38	T/S	N/A	–

(continued)

Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	USA	NCT01810913	Recruiting	II,III		**Arm1**: RT, Cisplatin **Arm2**: RT, Docetaxel **Arm3**: RT, Docetaxel, Cisplatin (Randomized)	675	DFS, OS	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	USA	NCT01824823	Recruiting	II		**Arm1**: Afatinib **Arm2**: Placebo (Randomized)	108	DFS	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	Asian countries	NCT01856478	Recruiting	III		**Arm1**: Afatinib **Arm2**: Methotrexate (Randomized)	300	DFS	N/A	–
EGFR TKI	Nilotinib	USA	NCT01871311	Recruiting	I		**Arm**: Nilotinib, Cetuximab (Single arm)	22	T/S	N/A	–
EGFR MoAb	Cetuximab	UK	NCT01874171	Recruiting	III		**Arm1**: Cetuximab, RT **Arm2**: Cisplatin, RT (Randomized)	304	T/S	N/A	–
EGFR MoAb	Cetuximab	France	NCT01884623	Recruiting	III		**Arm1**: Cetuximab **Arm2**: Methotrexate (Randomized)	164	FFS	N/A	–
EGFR MoAb	Cetuximab	Sweden	NCT01969877	Recruiting	III		**Arm1**: RT, Cetuximab **Arm2**: RT, Cisplatin (Randomized)	618	OS	N/A	–
MKI	Sorafenib	USA	NCT02035527	Recruiting	II		**Arm**: Sorafenib, Cisplatin, Docetaxel (Single arm)	41	T/S, PFS	N/A	–
EGFR MoAb	CetuGEX™	Germany and others	NCT02052960	Recruiting	II		**Arm1**: CetuGEX™, Cisplatin, 5-Fluorouracil **Arm2**: Cetuximab, Cisplatin, 5-Fluorouracil (Randomized)	240	PFS	N/A	–

(continued)

Table 10.1 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms): Radiation (RT), Surgery (Surg)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
EGFR MoAb	Cetuximab	Netherlands	NCT02054442	Recruiting	I,II		**Arm1**: Cetuximab, Methotrexate **Arm2**: Methotrexate (Randomized)	120	DLT,PFS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT02057107	Recruiting	II		**Arm1**: RT, Cetuximab, Docetaxel **Arm2**: RT, Cetuximab (Randomized)	92	PFS	N/A	–
EGFR MoAb	Cetuximab	USA	NCT02128906	Recruiting	II		**Arm1**: Docetaxel, Cetuximab, IMRT **Arm2**: Cisplatin, IMRT (Randomized)	160	TTP	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	China, Korea, Republic of Singapore and Taiwan	NCT02131155	Recruiting	III		**Arm1**: Afatinib **Arm2**: Placebo (Randomized)	150	DFS	N/A	–
Dual TKI (EGFR/HER2)	Afatinib	France	NCT02216617	Recruiting	I		**Arm**: Taxotere, Cisplatin, Afatinib (Single arm)	22	MTD	N/A	–
EGFR MoAb	Cetuximab	France, Germany and Spain	NCT02268695	Recruiting	II		**Arm1**: Cisplatin, 5-FU, Cetuximab **Arm2**: Cisplatin, Docetaxel, Cetuximab, G-CSF (Randomized)	416	OS	N/A	–

Overall survival (OS), Disease free survival (DFS), Event-free survival (EFS), Progression free survival (PFS), Local regional control (LRC), Toxicity/ Safety (T/S), Adverse event (AE), Maximum tolerated dose (MTD), Completion rate (CR), Pathology (P), Pharmacokinetics (PK), Compliance (C), Rate of disease control (RDC), Time to progression (TTP), Failure free survival (FFS), Overall response rate (ORR) and Tumor resolution (TR)

SD stable disease; *GR* good response; *NR* non-response; *PD* progression disease; *CR* complete response; *PR* partial response; *N/A* not applicable; *HR* hazard ratio; *MoAb* monoclonal antibody; *TKI* tyrosine kinase inhibitor; *MKI* multitargeted kinase inhibitor

Table 10.2 Summary of clinical trials for molecular target therapy on oral/head and neck squamous cell carcinoma (other than EGFR inhibitors)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
Adenovirus gene therapy	INGN 201 (Ad5CMV-p53)	USA	NCT00017173	Completed	II	Newly diagnosed, previously untreated SCCHN (stage III or IV)	**Arm**: INGN 201 (Ad5CMV-p53), Cisplatin, Surgery, Radiation (Single arm)	13	T/S	Grade 4 AE: 20%, grade 3 AE: 70%.	Yoo et al. (2009)
Adenovirus gene therapy	INGN 201	USA	NCT00064103	Completed	I,II		**Arm**: Ad5CMV-p53 (Single arm)	51	T/S	No study results posted	–
Proteasome inhibitor	Bortezonib	USA	NCT00103259	Completed	II	Recurrent or metastatic SCCHN	**Arm1**: Bortezonib, Irinotecan **Arm2**: Bortezonib (Randomized)	71	LRC	No CR in both Arms. PR: 13.1% (95%CI; 3.6–30.3) in Arm1 and 2.6% (95%CI; 0.4–22.1) in Arm2	Gilbert et al. (2013)
Adenovirus gene therapy	INGN 201	USA	NCT00410865	Terminated	I		**Arm**: INGN 201 (Single arm)	4	MTD	No study results posted	–
Proteasome inhibitor	Bortezonib	USA	NCT00425750	Completed	II	Recurrent or metastatic SCCHN	**Arm**: Bortezonib, Docetaxel (Single arm)	25	OS	PR: 5%, SD: 48% and PD: 48%	Chung et al. (2010)
Src/Abl kinase inhibitor	Saracatinib (AZD0530)	USA	NCT00513435	Completed	II	Recurrent or metastatic SCCHN	**Arm**: AZD0530 (Single arm)	9	LRC	SD: 11% and PD: 89%	Fury et al. (2011)
IGF-1R inhibitor	Cixutumumab (IMC-A12)	USA	NCT00617734	Completed	II		**Arm1**: IMC-A12 **Arm2**: IMC-A12, Cetuximab (Randomized)	91	OFS	No study results posted	–
MET/VEGFR inhibitor	Foretinib: GSK1363089 (XL880)	USA	NCT00725764	Completed	II	Refractory currable recurrent and/or metastatic SCCHN	**Arm**: GSK1363089 (Single arm)	14	LRC	SD: 50%, tumor shrinkage: 43% and prolonged disease stabilization for $\geq$13 months: 14%	Seiwert et al. (2013)
CDK inhibitor	P276-00	India	NCT00824343	Completed	II		**Arm**: P276-00 (Single arm)	86	LRC	No study results posted	–

(continued)

Table 10.2 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
mTOR inhibitor	Everolimus (RAD001)	USA	NCT00858663	Completed	I	SCCHN (stage III to IVB)	**Arm**: Radiation therapy, RAD001 (everolimus), Cisplatin (Single arm)	13	T/S	The common grade ≥ 3 treatment-related AEs: lymphopenia (92%) followed by mucositis.	Fury et al. (2013a)
Bcr-Abl TKI	Dasatinib	USA	NCT00882583	Active, not recruiting	I,II		**Arm1**: Dasatinib, Cetuximab, Radiation **Arm2**: Dasatinib, Cisplatin, Cetuximab, Radiation (Randomized)	98	T/S, LRC	No study results posted	–
CDK inhibitor	P276-00	India	NCT00899054	Completed	I,II		**Arm**: P276-00, Radiation (Single arm)	23	T/S	No study results posted	–
mTOR inhibitor	Everolimus (RAD002)	USA	NCT00935961	Completed	I	Previously untreated SCCHN (stage III to IVB)	**Arm**: RAD001, Docetaxel, Cisplatin (Single arm)	18	T/S	The most common grade 3 or 4 AEs: leukopenia and lymphopenia.	Fury et al. (2013b)
IGF-1R inhibitor	Cixutumumab (IMC-A12)	USA	NCT00957853	Recruiting	II		**Arm1**: Cetuximab **Arm2**: IMC-A12 **Arm3**: Cetuximab, IMC-A12 (Randomized)	60	P	No study results posted	–
mTOR inhibitor	Temsirolimus	USA	NCT01009203	Completed	II	Recurrent and/or metastatic, platinum-refractory SCCHN	**Arm**: Temsirolimus, Erlotinib (Single arm)	13	PFS	Median PFS: 1.9 months	Bauman et al. (2013)
mTOR inhibitor	Everolimus (RAD001)	USA	NCT01009346	Completed	I		**Arm**: RAD001, Cetuximab, Cisplatin, Carboplatin (Single arm)	9	PFS	PFS: 2.8 months (95%CI: 1.6–13.9)	–

(continued)

Table 10.2 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
mTOR inhibitor	Temsirolimus	USA	NCT01015664	Completed	I,II		**Arm**: Temsirolimus, cisplatin, cetuximab (Single arm)	11	T/S, PFS	No study results posted	–
Aurora A kinase inhibitor	Alisertib (MLN8237)	USA	NCT01045421	Recruiting	I,II	Breast cancer, small-cell lung cancer, non-small-cell lung cancer, SCCHN, and gastro-oesophageal adenocarcinoma	**Arm**: MLN8237 (Single arm)	273	T/S, OS	No study results posted	Melichar et al. (2015)
mTOR inhibitor	Everolimus (RAD001)	USA	NCT01057277	Completed	I		**Arm**: RAD001(Afinitor) Rad001, Cisplatin, Radiation (Single arm)	3	LRC	No study results posted	–
mTOR inhibitor	Everolimus (RAD001)	USA	NCT01058408	Completed	I		**Arm**: Radiotherapy, RAD001, Cisplatin (Single arm)	3	T/S	No study results posted	–
mTOR inhibitor	Everolimus (RAD001)	USA	NCT01111058	Recruiting	II		**Arm1**: Everolimus (RAD 001) **Arm2**: Placebo (Randomized)	160	PFS	No study results posted	–
Immunomodulatory drugs (IMiDs)	Lenalidomide	USA	NCT01133665	Completed	II		**Arm**: Cetuximab, Lenalidomide (Single arm)	40	PFS,P	PFS: 1.8 (median months) (1.0–11.7)	–
mTOR inhibitor	Everolimus (RAD001)	USA	NCT01133678	Recruiting	II	Locally advanced HNSCC	**Arm1**: Everolimus, Cisplatin, Paclitaxel, Cetuximab **Arm2**: Everolimus or Placebo, Cisplatin, Paclitaxel, Cetuximab (Randomized)	80	LRC	No study results posted	Villaflor et al. (2016)

(continued)

Table 10.2 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
PDK inhibitor	Dichloroacetate	USA	NCT01163487	Recruiting	I		**Arm**: Dichloroacetate (Single arm)	18	MTD	No study results posted	–
mTOR inhibitor	Temsirolimus	Germany	NCT01172769	Completed	II	Platin- and cetuximab refractory/recurrent and/or metastatic SCCHN	**Arm**: Temsirolimus (Single arm)	42	PFR	PFR at 12 weeks: 40% (95%CI 25.0–54.6)	Grünwald et al. (2015)
mTOR inhibitor	Rapamycin	USA	NCT01195922	Recruiting	I,II		**Arm**: Rapamycin (Single arm)	50	LRC	No study results posted	–
mTOR inhibitor	Ridaforolimus	USA	NCT01212627	Completed	I		**Arm**: Ridaforolimus (Single arm)	12	T/S	No study results posted	–
Hedgehog Inhibitor	Saridegib (IPI-926)	USA	NCT01255800	Completed	I		**Arm**: IPI-926, Cetuximab (Single arm)	9	T/S	No study results posted	–
mTOR inhibitor	Temsirolimus	USA	NCT01256385	Active, not recruiting	II		**Arm1**: Cetuximab, Temsirolimus **Arm2**: Temsirolimus (Randomized)	80	PFS	No study results posted	–
MET inhibitor	LY2801653	USA	NCT01285037	Recruiting	I		**Arm**: LY2801653, Cetuximab, Cisplatin (Single arm)	160	T/S	No study results posted	–
mTOR inhibitor	Everolimus	UK	NCT01313390	Completed	I,II		**Arm1**: Docetaxel **Arm2**: Docetaxel, Everolimus (Randomized)	4	T/S, MTD	No study results posted	–
mTOR inhibitor	Everolimus	USA	NCT01332279	Completed	I		**Arm**: Everolimus, Erlotinib, Radiation (Single arm)	40	MTD	No study results posted	–
AMPK activator	Metformin	Brazil	NCT01333852	Recruiting	II		**Arm1**: Metformin, Paclitaxel **Arm2**: Paclitaxel, Placebo (Randomized)	45	LRC	No study results posted	–

(continued)

Table 10.2 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
AKT inhibitor	MK2206	USA	NCT01349933	Completed	II		**Arm**: MK2206 (Single arm)	21	PFS	No study results posted	–
ALK1 inhibitor	Dalantercept	USA	NCT01458392	Active, not recruiting	II		**Arm**: Dalantercept (Single arm)	45	LRC	No study results posted	–
P13K inhibitor	BYL719	USA	NCT01602315	Recruiting	I,II		**Arm1**: Phase Ib: A-BYL719, cetux **Arm2**: Phase II: 2-Cetuximab **Arm3**: Phase Ib: B-BYL719, cetux **Arm4**: Phase II: 3-BYL719, Cetuximab **Arm5**: Phase II: 1-BYL719, Cetuximab **Arm6**: Phase Ib: C-BYL719 DT, cetuximab (Randomized)	202	T/S, PFS	No study results posted	–
P13K inhibitor	BKM120	France	NCT01737450	Recruiting	II		**Arm**: BKM120 (Single arm)	70	LRC	No study results posted	–
PARP-1 inhibitor	Olaparib	USA	NCT01758731	Recruiting	I		**Arm**: Olaparib, Cetuximab, Radiation (Single arm)	24	T/S	No study results posted	–
P13K inhibitor	BKM120	USA	NCT01816984	Recruiting	I,II		**Arm1**: BKM120, Cetuximab **Arm2**: BKM120 (without initial treatment), Cetuximab (Randomized)	30	P, MTD	No study results posted	–
Toll-like receptor (TLR) 8 agonist	VTX-2337	USA	NCT01836029	Recruiting	II		**Arm1**: Chemotherapy, Cetuximab, VTX-2337 **Arm2**: Chemotherapy, Cetuximab, Placebo (Randomized)	175	PFS	No study results posted	–

(continued)

Table 10.2 (continued)

Type of drug	Drug	Setting	NCT number	Study status	Study phase	Patients (publications only)	Study design (Description of arms)	# of patients	Primary objectives (outcome measures)	Study outcomes (posted in ClinicalTrials.gov)	Publication status (citation)
P13K inhibitor	BKM120	USA and others	NCT01852292	Recruiting	II		**Arm1**: Paclitaxel, Buparlisib (BKM120) **Arm2**: Paclitaxel, Placebo (Randomized)	150	PFS	No study results posted	–
Hypoxic radiosensitizer	Nimorazole	UK	NCT01950689	Recruiting	III		**Arm1**: Nimorazole, Radiation **Arm2**: Radiation (Randomized)	470	LRC	No study results posted	–
P13K inhibitor	BKM120	USA	NCT02113878	Recruiting	I		**Arm**: BKM120, Cisplatin, Radiation (Single arm)	46	MTD	No study results posted	–
RTK inhibitor	MGCD516	USA	NCT02219711	Recruiting	I		**Arm**: MGCD516 (Single arm)	120	AE	No study results posted	–
PARP-1 inhibitor	Olaparib	Netherland	NCT02229656	Recruiting	I		**Arm**: Radiotherapy, Olaparib (Single arm)	36	T/S	No study results posted	–
P13K inhibitor	BYL719	USA	NCT02282371	Recruiting	I		**Arm**: Cetuximab, BYL719, Radiation (Single arm)	18	T/S	No study results posted	–

Overall survival (OS), Disease free survival (DFS), Event-free survival (EFS), Progression free survival (PFS), Local regional Control (LRC), Toxicity/ Safety (T/S), Adverse event (AE), Maximum tolerated dose (MTD), Pathology (P), Pharmacokinetics (PK), Compliance (C), rate of disease control (RDC), Time to progression (TTP), Failure free survival (FFS), Completion rate (CR)

SD stable disease; *GR* good response; *NR* non-response; *PD* progression disease; *CR* complete response; *PR* Partial response; *N/A* not applicable; *HR* hazard ratio, *ALK* anaplastic lymphoma kinase; *AMPK:AMP* activated protein kinase; *CDK* cyclin-dependent kinase; *mTOR* mammalian target of rapamycin; *PARP1* poly(ADP) ribose polymerase 1; *RTK* receptor tyrosine kinase; *PDK* pyruvate dehydrogenase kinase

We evaluated the outcomes of targeted therapies based on the primary outcome measures of the study results posted on the ClinicalTrials.gov or by examining respective publications.

The primary objectives are important for any clinical trial. Primary outcome definitions and results were largely concordant between Clinical Trials.gov and the Food and Drug Administration (FDA) approved drug products (Schwartz et al. 2016).

The primary objectives (outcome measures) in 63 trials (33%) were related to survival (OS, DFS, EFS, PFS and FFS), 57 (30%) related to adverse events (AE, C, T/S, CR and MTD), 55 (28%) related to responses (LRC, CR, PFR, ROC, ORR and TTP) and 18 (9%) related to others (P, PK).

60 out of 181 trials (33%) were published. Among published studies 17 (28%) did not post study results on the ClinicalTrials.gov. Overall, only 51 out of 181 (27%) studies posted their results on the ClinicalTrials.gov. A major problem is the lack of publication of the outcomes in large trials (Jones et al. 2013). Even some studies that had been published in various journals, no study results were posted in the database of ClinicalTrials.gov. To the contrary, some study results are posted in the trials registries, but were not published.

In Table 10.3, we list the systemic medications used in molecular targeted therapies in published trials. Some regimens include a combination with Cisplatin/Carboplatin, 5-FU or Docetaxel. Among EGFR inhibitors the largest number of trials have been the use of EGFR MoAb as either Cetuximab (n = 15) or Geftinib (9). Among Dual TKI (Tyrosine Kinase inhibitor) trials Bevacizumab has been used in 5 trials.

10.4 Summary of Published Studies

The summaries of published studies are given below classified by the target molecules. As said earlier, majority of the trials have examined the benefits of inhibition of EGFR.

10.4.1 EGFR inhibitors

EGFR MoAb - Cetuximab

Phase II study of pemetrexed in combination with cisplatin and cetuximab in recurrent or metastatic squamous cell carcinoma of the head and neck (Vermorken et al. 2013b). This was a multi-centre, open-label, single-arm study with the primary objective to estimate progression-free survival (PFS) in recurrent and metastatic head and neck cancer. Sixty-six patients received ≥ 1 cycle of the triplet. Most patients were male (80.3%), with a median age of 62 years and Eastern Cooperative Oncology Group (ECOG) performance status of 1 (71.2%). Diagnoses

Table 10.3 List of medications with dosage and number of trials for molecular target therapies on oral/head and neck squamous cell carcinoma (published and NCT number obtained)

Type of drugs		Drugs	Dosage	Regimens (combined drugs or treatment)	Number of trials (published)	Reference
EGFR inhibitors	EGFR MoAb	Cetuximab	250 mg/m^2 or 400 mg/m^2 weekly	Pemetrexed, Carboplatin/Cisplatin, Folic Acid Dietary, Vitamin B12 or EMD 1201081(IMO-2055) or Cetuximab, 5-FU or Cetuximab, Carboplatin, Paclitaxel, Surg, RT or Cetuximab, Cisplatin, RT or Cetuximab, Cisplatin/Carboplatin, 5-FU or RT or Cilengitide, 5-FU, Cisplatin	17	Vermorken et al. (2013b), Ruzsa et al. (2014), Vermorken et al. (2008), Kies et al. (2010), Egloff et al. (2014), Guo et al. (2015), Yoshino et al. (2013), Okano et al. (2013), Vermorken et al. (2014), Wanebo et al. (2014), Suntharalingam et al. (2012), Adkins et al. (2013), Magrini et al. (2016), Soulières et al. (2016), Bonner et al. (2006), Ang et al. (2014), Argini et al. 2010
		Bevacizumab	2.5–10 mg/kg	Erlotinib, Cisplatin, RT or Erlotinib, Paclitaxel, 5-FU, RT or Cetuximab or 5-FU, Hydroxyurea, RT or Erlotinib	5	Yoo et al. (2012), Hainsworth et al. (2011), Argiris et al. (2013a), Seiwert et al. (2008), Cohen et al. (2009)
		Zalutumumab	0.15–16 mg/kg	Single	3	Machiels et al. (2011), Bastholt et al. (2007), Saloura et al. (2014)
		Panitumumab	9 mg/kg on day 1 of each cycle (IV)	Cisplatin, 5-FU	3	Vermorken et al. (2013a), Mesía et al. (2015), Giralt et al. (2015)
		Sym004 (anti-EGFR antibody mixture)	12 mg/kg	Single	1	Machiels et al. (2015b)
	EGFR TKI	Gefitinib	250 mg/d or 500 mg/d, escalated to 750 mg/d	Docetaxel or Cisplatin/Carboplatin, 5-FU, Iressa or Docetaxel, Carboplatin, 5-FU, RT or Cisplatin, RT	9	Rodriguez et al. (2012), Gregoire et al. (2011), Perez et al. (2012), Van Waes et al. (2010), Argiris et al. (2013b), Hainsworth et al. (2009), Chen et al. (2007)
		Erlotinib	50 or 150 mg/d	Docetaxel, Cisplatin or Dasatinib or Sulindac	3	Kraut et al. (2011), Martins et al. (2013), Gross et al. (2014)
	Dual TKI	Lapatinib	1500 mg daily	Single or Fapatinib, Cisplatin, RT	3	Harrington et al. (2015), de Souza et al. (2012), Del Campo et al. (2011)
		Afatinib	40 mg/day	Single	1	Machiels et al. (2015a)
	MKI	Sunitinib	37.5 or 50 mg/d	Single	2	Choong et al. (2010), Machiels et al. (2010)
		Vandetanib (ZD6474)	100 mg/d	Docetaxel or Cisplatin, RT	2	Limaye et al. (2013), Papadimitrakopoulou et al. (2014)
	mTOR inhibitor	Everolimus (RAD002)	2.5–10 mg/d	Cisplatin, RT or Cisplatin, Docetaxel or Cisplatin, Paclitaxel, Cetuximab	3	Fury et al. (2013a), Fury et al. (2013b), Villaflor et al. (2016)

(continued)

Table 10.3 (continued)

Type of drugs		Drugs	Dosage	Regimens (combined drugs or treatment)	Number of trials (published)	Reference
Other targeted agents		Temsirolimus	15 mg or 25 mg weekly	Single or Erlotinib	2	Bauman et al. (2013), Grünwald et al. (2015)
	Proteasome inhibitor	Bortezonib	1.3 mg/m^2 or 1.6 mg/m^2	Docetaxel or Irinotecan	2	Chung et al. (2010), Gilbert et al. (2013)
	MET/VEGFR inhibitor	Foretinib: GSK1363089 (XL880)	240 mg/d	Single	1	Seiwert et al. (2013)
	Src/Abl kinase inhibitor	AZD0530 (Saracatinib)	175 mg/d	Single	1	Fury et al. (2011)
	Adenovirus gene therapy	INGN 202	Several doses injected into surgical beds or drain catheter perioperatively	Cisplatin, Surgery, RT	1	Yoo et al. (2009)
	Aurora A kinase inhibitor	Alisertib (MLN8237)	50 mg twice daily	Single	1	Melichar et al. (2015)

EGFR Epidermal growth factor receptor
MoAb Monoclonal antibody
RT Radiotherapy
TKI Tyrosine-kinase inhibitor
MKI Multitargeted kinase inhibitors
VEGFR Vascular endothelial growth aspect receptors
mTOR Mammalian target of rapamycin
MET Mesenchymal epithelial transition
NCT ClinicalTrials.gov registry number

included oropharynx (45.5%) and larynx (24.2%) cancers, with locoregional disease (51.5%) alone, or combined with distant metastases (48.5%). Median (m) PFS was 4.4 months (95% confidence interval [CI]: 3.6, 5.4); median overall survival was 9.7 months (95% CI: 6.5, 13.1). There were eight deaths on treatment. Objective response rate was 29.3%; 23 patients had stable disease (39.7%). Drug-related grade 3/4 toxicities included neutropaenia (33.3%), fatigue (24.2%), anorexia (12.1%) and infection (10.6%). Five treatment-related deaths (7.6%) occurred.

Phase 2, open-label, 1:1 randomized controlled trial exploring the efficacy of EMD 1201081 in combination with cetuximab in second-line cetuximab-naïve patients with recurrent or metastatic squamous cell carcinoma of the head and neck (R/M SCCHN) (Ruzsa et al. 2014). Objective response rate in both arms was 5.7% (95% CI 1.2–15.7%) by independent assessment. Disease control was 37.7% for patients on combination (24.8–52.1%) and 43.4% on control (29.8–57.7%). Neither independent nor investigator assessments showed significant differences between study arms. Median progression-free survival was 1.5 months (1.3–2.6) for patients on combination, and 1.9 months (1.5–2.9) on control. The most frequent adverse events in the combination arm were rash (29.6%), acneiform dermatitis (22.2%), and injection site reactions (20.4%). Grade 3/4 dyspnea and hypokalemia were more frequent with cetuximab monotherapy (7.5 and 5.7% vs. 1.9% each, respectively), and grade 3/4 respiratory failure and disease progression were more frequent with combination (5.6% each vs. 1.9% each).

Platinum-based chemotherapy plus cetuximab in head and neck cancer (Vermorken et al. 2008). This was a multi-centre randomized clinical trial run in 17 European countries. Patients received a maximum of six cycles of chemotherapy, receiving either cisplatin or carboplatin and an infusion of fluorouracil every 3 weeks. Cetuximab was given as an initial 2 h intravenous infusion dose of 400 mg/sq meter), followed by weekly doses of 1 h IV infusion (dose 250 mg per square meter). Adding cetuximab to platinum-based chemotherapy with fluorouracil (platinum-fluorouracil) significantly prolonged the median overall survival from 7.4 months in the chemotherapy-alone group to 10.1 months in the group that received chemotherapy plus cetuximab (hazard ratio for death, 0.80; 95% confidence interval, 0.64–0.99; $P = 0.04$). The addition of cetuximab prolonged the median progression-free survival time from 3.3 to 5.6 months (hazard ratio for progression, 0.54; $P < 0.001$) and increased the response rate from 20 to 36% ($P < 0.001$). The most common grade 3 or 4 adverse events in the chemotherapy-alone and cetuximab groups were anemia (19 and 13%, respectively), neutropenia (23 and 22%), and thrombocytopenia (11% in both groups). Sepsis occurred in 9 patients in the cetuximab group and in 1 patient in the chemotherapy-alone group ($P = 0.02$). Of 219 patients receiving cetuximab, 9% had grade 3 skin reactions and 3% had grade 3 or 4 infusion-related reactions. There were no cetuximab-related deaths.

Induction chemotherapy and cetuximab for locally advanced squamous cell carcinoma of the head and neck: results from a phase II prospective trial (Kies et al. 2010). After induction PCC, nine patients (19%) achieved a complete response, and 36 patients (77%) achieved a partial response. The most common

grade 3 or 4 toxicity was skin rash (45%), followed by neutropenia (21%) without fever. At a median follow-up time of 33 months, locoregional or systemic disease progression was observed in six patients. The 3-year progression-free survival (PFS) and overall survival (OS) rates were 87% (95% CI, 78–97%) and 91% (95% CI, 84–99%), respectively. Human papillomavirus (HPV) 16, found in 12 (46%) of 26 biopsies, was associated with improved PFS ($P = 0.012$) and OS ($P = 0.046$).

Phase II Study of Cetuximab in Combination with Cisplatin and Radiation in Unresectable, Locally Advanced Head and Neck Squamous Cell Carcinoma: Eastern Cooperative Oncology Group Trial E3303 (Egloff et al. 2014). A total of 69 patients were enrolled; 60 proved eligible and received protocol treatment. Oropharyngeal primaries constituted the majority (66.7%), stage T4 48.3% and N2–3 91.7%. Median radiotherapy dose delivered was 70 Gy, 71.6% received all three cycles of cisplatin, and 74.6% received maintenance cetuximab. Median PFS was 19.4 months, 2-year PFS 47% [95% confidence interval (CI), 33–61%]. Two-year overall survival (OS) was 66% (95% CI, 53–77%); median OS was not reached. Response rate was 66.7%. Most common grade ≥ 3 toxicities included mucositis (55%), dysphagia (46%), and neutropenia (26%); one attributable grade 5 toxicity occurred. Only tumor HPV status was significantly associated with survival. HPV was evaluable in 29 tumors; 10 (all oropharyngeal) were HPV positive. HPV (+) patients had significantly longer OS and PFS ($P = 0.004$ and $P = 0.036$, respectively).

Platinum-based chemotherapy plus cetuximab first-line for Asian patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck (Guo et al. 2015). Results of an open-label, single-arm, multicenter trial. The overall response rate was 55.9%, including 2 complete responses (CRs). Median overall survival (OS) was 12.6 months and median progression-free survival (PFS) was 6.6 months. Grade 3/4 adverse events (AEs) were reported in 41 (60.3%) patients. The safety profile was in line with previous clinical experience. The pharmacokinetic profile was in line with that observed with cetuximab in white and Japanese patients.

Platinum-based chemotherapy plus cetuximab for the first-line treatment of Japanese patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck: results of a phase II trial (Yoshino et al. 2013). In total, 33 patients received treatment. The most frequent primary tumor site was the hypopharynx (42%), and most patients had metastatic disease (85%). The best overall response rate as assessed by the independent review committee was 36% (95% confidence interval: 20, 55) and was significantly greater ($P = 0.002$) than the protocol-specified threshold of 15% at the one-sided 5% level. The disease control rate was 88%. The median progression-free survival and overall survival were 4.1 and 14.1 months, respectively. There were no unexpected safety concerns. Grade 3 or 4 adverse events were experienced by nearly all patients (32, 97%). No adverse events were fatal.

Phase II study of cetuximab plus concomitant boost radiotherapy in Japanese patients with locally advanced squamous cell carcinoma of the head and neck (Okano et al. 2013). Twenty-two patients were evaluable. The treatment

completion rate was 100% (95% confidence interval 85–100). The response rate 8 weeks post-radiotherapy was 82% (95% confidence interval 60–95). The most common grade 3/4 treatment-emergent adverse events were mucosal inflammation (73%); dermatitis (27%); and infection, radiation skin injury and stomatitis (23% each).

Cisplatin, 5-fluorouracil, and cetuximab (PFE) with or without cilengitide in recurrent/metastatic squamous cell carcinoma of the head and neck: results of the randomized phase I/II ADVANTAGE trial (phase II part) (Vermorken et al. 2014). One hundred and eighty-two patients were treated. Median PFS per investigator read was similar for CIL1 W + PFE, CIL2 W + PFE, and PFE alone (6.4, 5.6, and 5.7 months, respectively). Accordingly, median overall survival and objective response rates were not improved with cilengitide (12.4 months/47%, 10.6 months/27%, and 11.6 months/36%, respectively). No clinically meaningful safety differences were observed between groups. None of the tested biomarkers (expression of integrins, CD31, Ki-67, vascular endothelial growth factor receptor 2, vascular endothelial-cadherin, type IV collagen, epidermal growth factor receptor, or p16 for human papillomavirus) were predictive of outcome.

Induction cetuximab, paclitaxel, and carboplatin followed by chemoradiation with cetuximab, paclitaxel, and carboplatin for stage III/IV head and neck squamous cancer: a phase II ECOG-ACRIN trial (E2303) (Wanebo et al. 2014). Seventy-four patients were enrolled; 63 were eligible. Forty-four (70%) were free of surgery to the primary site, progression, and death 1-year post-treatment. Following induction, 41 (23 CR) underwent week 8 primary site biopsy and 24 (59%) had no tumor (pathologic CR). Week 14 biopsy during chemoradiation (50 Gy) in 34 (15 previously positive biopsy; 19 no prior biopsy) was negative in 33. Thus 90% of eligible patients completed CRT. Overall survival and EFS were 78 and 55% at 3 years, respectively. Disease progression in 23 patients (37%) was local only in 10 (16%), regional in 5 (8%), local and regional in 2 (3%), and distant in 5 patients (8%). There were no treatment-related deaths. Toxicity was primarily hematologic or radiation-related. p16 AQUA score was not associated with response/survival.

Phase II study evaluating the addition of cetuximab to the concurrent delivery of weekly carboplatin, paclitaxel, and daily radiotherapy for patients with locally advanced squamous cell carcinomas of the head and neck (Suntharalingam et al. 2012). All patients completed the planned RT dose, 74% without any treatment breaks. The planned CTX and PC cycles were completed in 70% (91% with at least seven of planned nine cycles) and 56% (93% with at least seven of planned eight cycles) of patients, respectively. Toxicity included Grade 3 mucositis (79%), rash (9%), leucopenia (19%), neutropenia (19%), and RT dermatitis (16%). The complete response (CR) rate at the completion of therapy was 84%. The estimated 3-year local regional control rate was 72%. Six patients with an initial CR subsequently experienced a local recurrence, 10 patients experienced distant progression. The median overall survival and disease-free survivals have not been reached. The 3-year actuarial overall survival and disease-free survival were 59 and 58%, respectively.

A phase 2 trial of induction nab-paclitaxel and cetuximab given with cisplatin and 5-fluorouracil followed by concurrent cisplatin and radiation for locally advanced squamous cell carcinoma of the head and neck (Adkins et al. 2013). Thirty patients were enrolled, of which 22 (73%) had large (T3/T4) primary tumors. The CR rate at the primary tumor site after 2 cycles of ACPF was 53% and the overall response rate was 100%. Twenty-nine (96%) patients completed 3 cycles of ACPF, 26 (90%) completed definitive RT per protocol, and 22 of the 27 evaluable patients (81%) received >2 of the 3 planned doses of cisplatin with RT. The estimated 2-year overall and progression-free survival rates were 84 and 65%, respectively.

Cetuximab and Radiotherapy Versus Cisplatin and Radiotherapy for Locally Advanced Head and Neck Cancer: A Randomized Phase II Trial (Magrini et al. 2016). The study was discontinued early because of slow accrual after the enrollment of 70 patients. RT discontinuation for more than 10 days occurred in 13% of patients given CTX and 0% given CDDP ($P = 0.05$). Drug dosage reduction occurred in 34% given CTX and 53% given CDDP (difference not significant). Toxicity profiles differed between the two arms, with hematologic, renal, and GI toxicities more frequent in the CDDP arm, and cutaneous toxicity and the need for nutritional support more frequent in the CTX arm. Serious adverse events related to treatment, including four versus one toxic deaths, were higher in the CTX arm (19% vs. 3%, $P = 0.044$). Locoregional control, patterns of failure, and survivals were similar between the treatment arms.

Cetuximab plus platinum-based chemotherapy in head and neck squamous cell carcinoma: a randomized, double-blind safety study comparing cetuximab produced from two manufacturing processes using the EXTREME study regimen (Soulières et al. 2016). The majority of patients experienced ≥ 1 TEAE, regardless of causality (Arm A: 75/77 patients, 97.4%; Arm B: 68/71 patients, 95.8%). TEAEs with the highest incidence included nausea, fatigue, and hypomagnesemia in both arms. The absolute risk difference between the two arms for patients experiencing at least one adverse event (AE) was 0.029 ($p = 0.281$, 95% confidence interval [CI]: -0.024, 0.082) for AEs regardless of causality and 0.005 ($p = 0.915$, 95% CI: −0.092, 0.103) for AEs possibly related to study drug. There were no significant differences between the two arms in the incidence of acneiform rash, cardiac events, infusion reactions, or hypomagnesemia. Overall survival, progression-free survival, and overall response rates were similar in the two arms.

Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck (Bonner et al. 2006). The median duration of locoregional control was 24.4 months among patients treated with cetuximab plus radiotherapy and 14.9 months among those given radiotherapy alone (hazard ratio for locoregional progression or death, 0.68; $P = 0.005$). With a median follow-up of 54.0 months, the median duration of overall survival was 49.0 months among patients treated with combined therapy and 29.3 months among those treated with radiotherapy alone (hazard ratio for death, 0.74; $P = 0.03$). Radiotherapy plus cetuximab significantly prolonged progression-free survival (hazard ratio for disease progression or death, 0.70; $P = 0.006$). With the exception of acneiform rash and infusion

reactions, the incidence of grade 3 or greater toxic effects, including mucositis, did not differ significantly between the two groups.

Randomized phase III trial of concurrent accelerated radiation plus cisplatin with or without cetuximab for stage III to IV head and neck carcinoma: RTOG 0522 (Ang et al. 2014). Of 891 analyzed patients, 630 were alive at analysis (median follow-up, 3.8 years). Cetuximab plus cisplatin-radiation, versus cisplatin-radiation alone, resulted in more frequent interruptions in radiation therapy (26.9% vs. 15.1%, respectively); similar cisplatin delivery (mean, 185.7 mg/m^2 vs. 191.1 mg/m^2, respectively); and more grade 3–4 radiation mucositis (43.2% vs. 33.3%, respectively), rash, fatigue, anorexia, and hypokalemia, but not more late toxicity. No differences were found between arms A and B in 30-day mortality (1.8% vs. 2.0%, respectively; $P = 0.81$), 3-year PFS (61.2% vs. 58.9%, respectively; $P = 0.76$), 3-year OS (72.9% vs. 75.8%, respectively; $P = 0.32$), locoregional failure (19.9% vs. 25.9%, respectively; $P = 0.97$), or distant metastasis (13.0% vs. 9.7%, respectively; $P = 0.08$). Patients with p16-positive oropharyngeal carcinoma (OPC), compared with patients with p16-negative OPC, had better 3-year probability of PFS (72.8% vs. 49.2%, respectively; $P < 0.001$) and OS (85.6% vs. 60.1%, respectively; $P < 0.001$), but tumor epidermal growth factor receptor (EGFR) expression did not distinguish outcome.

Induction docetaxel, cisplatin, and cetuximab followed by concurrent radiotherapy, cisplatin, and cetuximab and maintenance cetuximab in patients with locally advanced head and neck cancer (Argiris et al. 2010). Of 39 enrolled patients, 36 had stage IV disease and 23 an oropharyngeal primary. Acute toxicities during TPE included neutropenic fever (10%) and during XPE, grade 3 or 4 oral mucositis (54%) and hypomagnesemia (39%). With a median follow-up of 36 months, 3-year progression-free survival and overall survival were 70 and 74%, respectively. Eight patients progressed in locoregional sites, three in distant, and one in both. HPV positivity was not associated with treatment efficacy. No progression-free patient remained G-tube dependent. The H&N subscale QOL scores showed a significant decrement at 3 months after XPE, which normalized at 1 year.

EGFR MoAb - Zalutumumab

Reported on Zalutumumab plus best supportive care versus best supportive care alone in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy. This was an open-label, randomised phase 3 multi-centre trial on patients incurable with standard therapy (Machiels et al. 2011). The authors randomly allocated 191 (67%) of 286 eligible patients (stratified by performance status) to the zalutumumab group and 95 (33%) to the control group who received best supportive care. The primary end point was overall survival. Zalutumumab dose was titrated on the basis of evolution of a rash. Median overall survival in the test group was 6.7 months (95% CI 5.8–7.0) and 5.2 months (4.1–6.4) in the control group. The hazard ratio for death was between the patients in the zalutumumab group and controls stratified by WHO performance status was 0.77 (97% CI 0.57–1.05). Although zalutumumab did not increase overall survival, progression-free survival was significantly

Table 10.4 Common adverse events (toxicities) per each drug regimen for molecular target therapy

Toxicity	Drug/dose		Frequency/grade (3 or more grade)	Reference
Skin rash	Gefitinib	Single (250–750 mg/d)	16%/grade 3	Perez et al. (2012)
	Panitumumab	Panitumumab (9 mg/kg) with Cisplatin, 5-FU	19%/grade 3/4	Vermorken et al. (2013a)
	Erlotinib	Erlotinib (50 mg/d) with Docetaxel	44%/grade 3/4	Kraut et al. (2011)
	Cetuximab	Cetuximab (Initial dose of 250 mg/m^2 or 400 mg/m^2, followed by subsequent weekly doses of 250 mg/m^2) with cisplatin, 5-FU or Carboplatin/Cisplatin, Folic Acid Dietary, Vitamin B12 or Carboplatin, Paclitaxel, Surg, RT	9, 15, 27, 44 or 45%/grade 3/4	Vermorken et al. (2013a), Vermorken et al. (2008), Kies et al. (2010), Okano et al. (2013), Magrini et al. (2016), Ang et al. (2014)
	Zalutumumab	Single (0.15–16 mg/kg)	21%/grade 3/4	Machiels et al. (2011)
	Sym004	Single (12 mg/kg)	50%/grade 3	Machiels et al. (2015b)
Acneiform rash	Cetuximab	Cetuximab (250 mg/m^2) with Cisplatin, 5-FU, RT or Cetuximab, Pemetrexed, Carboplatin or Cisplatin, RT	12, 14, 15, 24%/grade 3/4	Vermorken et al. (2013b), Egloff et al. (2014), Yoshino et al. (2013), Okano et al. (2013), Adkins et al. (2013)
Mucositis	Gefitinib	Gefitinib (250 or 400 mg/d) with cisplatin, 5-FU, Iressa, RT or Paclitaxel, RT or Cisplatin, RT or Docetaxel, Carboplatin, 5-FU, RT or Docetaxel, RT	27–87%/grade 3 or more	Rodriguez et al. (2012), Gregoire et al. (2011), Hainsworth et al. (2009), Chen et al. (2007), Bonner et al. (2006)
	Bevacizumab	Bevacizumab (10 mg/kg) with Erlotinib, Cisplatin, 5-FU, Hydroxyurea	50 or 73%/grade 3	Yoo et al. (2012), Seiwert et al. (2008)
	Everolimus	Everolimus (2.5–10 mg/d) with Cisplatin, RT	62% (functional)/grade 3 or more	Fury et al. (2013a)
	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Cisplatin, RT or Carboplatin, Paclitaxel, RT or RT	23, 52, 55, 59 or 79%/grade 3 or more	Egloff et al. (2014), Okano et al. (2013), Wanebo et al. (2014), Suntharalingam et al. (2012), Magrini et al. (2016), Ang et al. (2014), Argiris et al. (2010)
	Lapatinib	Single (1500 mg daily)	46%/grade 3/4	Ang et al. (2014)
Mucosal inflammation	Panitumumab	Panitumumab (9 mg/kg) with cisplatin, RT	55%/grade 3	Mesía et al. (2015)
Vomiting	INGN 201 (Ad5CMV-p53)	INGN 201 (Ad5CMV-p53) with Cisplatin, Surg, RT	23%/grade 3/4	Yoo et al. (2009)

(continued)

Table 10.4 (continued)

Toxicity	Drug/dose		Frequency/grade (3 or more grade)	Reference
Diarrhea	Vandetanib	Vandetanib (100 mg or 200 mg) with Cisplatin, RT	85%/grade 3	Papadimitrakopoulou et al. (2014)
	Afatinib	Single (40 mg/d)	11%/grade 3/4 (aged $\geq$65 years)	Machiels et al. (2015a)
Gastrointestinal disorders	Zalutumumab	Single (4–16 mg/kg)	12%/grade 3/4	Saloura et al. (2014)
RT-GI mucositis, NOS	INGN 201 (Ad5CMV-p53)	INGN 201 (Ad5CMV-p53) with Cisplatin, Surg, RT	23%/grade 3	Yoo et al. (2009)
Fatigue	Foretinib	Single (240 mg/d)	14%/grade 3, 4 or 5	Seiwert et al. (2013)
	Everolimus	Everolimus (5–10 mg/d) with Cisplatin, Docetaxel	13%/grade 3	Fury et al. (2013b)
	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Pemetrexed, Carboplatin or Cisplatin, Folic Acid Dietary, Vitamin B12 or Cilengitide, 5-FU, Cisplatin or Carboplatin, Paclitaxel, Surg	17 or 24%/grade 3/4	Vermorken et al. (2013b), Vermorken et al. (2014), Ang et al. (2014), Argiris et al. (2010)
	Vandetanib	Vandetanib (100 mg/d or 200 mg/d) with Cisplatin, RT	85%/grade 3	Papadimitrakopoulou et al. (2014)
	Bortezomib	Bortezomib (1.3 mg/m^2) with Irinotecan	17%/grade 3	Gilbert et al. (2013)
	Sunitinib	Single (37.5 mg/d)	32%/grade 3/4	Machiels et al. (2010)
Dysphagia	Bevacizumab	Bevacizumab (2.5–10 mg/kg) with Erlotinib, Cisplatin, RT	30%/grade 3	Yoo et al. (2012)
	Everolimus	Everolimus (2.5–10 mg/d) with Cisplatin, RT	92%/grade 3 or more	Fury et al. (2013a)
	Cetuximab	Cetuximab (400 mg/m^2 initial dose followed by 250 mg/m^2) with Cisplatin, RT or Carboplatin, Paclitaxel, Surg or Carboplatin, Paclitaxel, RT	21 or 29%/grade 3/4	Wanebo et al. (2014), Suntharalingam et al. (2012), Bonner et al. (2006), Argiris et al. (2010)
	Gefitinib	Gefitinib (250 mg/d or 400 mg/d) with Iressa, Cisplatin, 5-FU, RT or Cisplatin, RT	39 or 68%/grade 3 or more	Rodriguez et al. (2012), Chen et al. (2007)

(continued)

Table 10.4 (continued)

Toxicity	Drug/dose		Frequency/grade (3 or more grade)	Reference
	Vandetanib	Vandetanib (100 mg/d or 200 mg/d) with Docetaxel or Cisplatin, RT	20 or 85%/grade 3 or 3/4	Limaye et al. (2013), Papadimitrakopoulou et al. (2014)
	Panitumumab	Panitumumab (9 mg/kg) with Cisplatin, RT	39%/grade 3	Mesía et al. (2015)
Infection	Zalutumumab	Single (4–16 mg/kg)	14%/grade 3/4	Saloura et al. (2014)
	Erlotinib	Erlotinib (150 mg/d) with Cisplatin, RT	46%/grade 3/4	Martins et al. (2013)
	Bortezomib	Bortezomib (1.6 mg/m^2) with Docetaxel	36%/grade 3	Chung et al. (2010)
Dehydration	Foretinib	Single (240 mg/d)	14%/grade 3, 4 or 5	Seiwert et al. (2013)
Neutropenia	Panitumumab	Panitumumab (9 mg/kg), Cisplatin, 5-FU,	32%/grade 3/4	Vermorken et al. (2013a)
	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Pemetrexed, Carboplatin or Cisplatin, Folic Acid Dietary, Vitamin B12 or Carboplatin, Paclitaxel, RT or Cisplatin, RT or Cilengitide, 5-FU, Cisplatin or Cisplatin, Carboplatin, 5-FU	8–79%/grade 3/4	Vermorken et al. (2013b), Vermorken et al. (2008), Kies et al. (2010), Egloff et al. (2014), Yoshino et al. (2013), Vermorken et al. (2014), Wanebo et al. (2014), Suntharalingam et al. (2012), Adkins et al. (2013)
	Gefitinib	Gefitinib (250 mg/d or 500 mg/d) with Iressa, Cisplatin, 5-FU, RT or Cisplatin, RT	30 or 77%/grade 3 or more	Rodriguez et al. (2012), Chen et al. (2007)
	Everolimus	Everolimus (5–10 mg/d) with Cisplatin, Docetaxel	40%/grade 3/4	Fury et al. (2013b)
	Vandetanib	Vandetanib (100 mg/d) with Docetaxel or Cisplatin, RT	40%/grade 3/4	Limaye et al. (2013)
	Alisertib (MLN8237)	Single (50 mg twice daily)	43%/grade 3/4	Melichar et al. (2015)
Hypomagnesaemia	Panitumumab	Panitumumab (9 mg/kg) with Cisplatin, 5-FU,	12%/grade 3/4	Vermorken et al. (2013a)
	Cetuximab	Cetuximab (400 mg/m^2 initial dose followed by 250 mg/m^2) with Cisplatin, 5-FU	9%/grade 3/4	Vermorken et al. (2008), Argiris et al. (2010)
	Sym004 (anti-EGFR antibody mixture)	Single (12 mg/kg)	38%/grade 3 or more	Machiels et al. (2015b)

(continued)

Table 10.4 (continued)

Toxicity	Drug/dose		Frequency/grade (3 or more grade)	Reference
Lymphopenia	Everolimus	Everolimus (2.5 or 5–10 mg/d) with Cisplatin or RT or Docetaxel	67 or 92%/grade 3 or more	Fury et al. (2013a), Fury et al. (2013b)
	INGN 201 (Ad5CMV-p53)	INGN 201 (Ad5CMV-p53) with Cisplatin, Surg, RT	23%/grade 3	Yoo et al. (2009)
	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Pemetrexed, Carboplatin or Cisplatin, Folic Acid Dietary, Vitamin B12	35%/grade 3/4	Vermorken et al. (2013b)
	Saracatinib	Single (175 mg/d)	22%/grade 3	Fury et al. (2011)
Leukopenia	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Cilengitide, 5-FU, Cisplatin or Carboplatin, Paclitaxel, Surg or Carboplatin, Paclitaxel, RT	21, 24 or 40%/grade 3/4	Vermorken et al. (2014), Wanebo et al. (2014), Suntharalingam et al. (2012)
	Alisertib (MLN8237)	Single (50 mg twice daily)	21%/grade 3/4	Melichar et al. (2015)
Anemia	Temsirolimus	Single (25 mg weekly)	15%/grade 3/4	Grünwald et al. (2015)
	Sunitinib	Single (37.5 mg/d)	11%/grade 3/4	Machiels et al. (2010)
Hyperglycemia	Everolimus	Everolimus (5–10 mg/d) with Cisplatin, Docetaxel	40%/grade 3/4	Fury et al. (2013b)
Hypokalemia	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Cilengitide, 5-FU, Cisplatin	19%/grade 3/4	Vermorken et al. (2014)
Dyspnea	Erlotinib	Erlotinib (150 mg/d) with Docetaxel	25%/grade 3/4	Bauman et al. (2013)
	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Cisplatin, RT	55%/grade 3 or more	Egloff et al. (2014), Argiris et al. (2010)
Respiratory failure	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with EMD 1201081(IMO-2055)	6%/grade 3/4	Ruzsa et al. (2014)
Sepsis (including septic shock)	Cetuximab	Cetuximab (250 mg/m^2 or 400 mg/m^2) with Cisplatin, 5-FU	7%/grade 3/4	Vermorken et al. (2008)
Asthenia	Erlotinib	Erlotinib (150 mg/d) with Docetaxel	42%/grade 3/4	Bauman et al. (2013)
Renal dysfunction	Gefitinib	Gefitinib (250 or 400 mg/d) with Iressa, Cisplatin, 5-FU, RT	28%/grade 3 or more	Rodriguez et al. (2012)

extended in patients with recurrent squamous-cell carcinoma of the head and neck who had failed platinum-based chemotherapy. The most common grade 3–4 adverse events were rash (39 [21%] patients in the zalutumumab group vs. none in the control group), anaemia (11 [6%] vs. five [5%]), and pneumonia (nine [5%] vs. two [2%]). 28 (15%) patients in the zalutumumab group had grade 3/4 infections compared with eight (9%) in the control group. The most common serious adverse events were tumour haemorrhage (28 [15%] patients given zalutumumab vs. 13 [14%] controls), pneumonia (13 [7%] vs. three [3%]), and dysphagia (11 [6%] vs. two [2%]).

This was a Phase I/II clinical and pharmacokinetic study evaluating a fully human monoclonal antibody against EGFr (HuMax-EGFr) in patients with advanced squamous cell carcinoma of the head and neck (Bastholt et al. 2007). The primary aim was to establish the safety profile of EGFr (HuMax-EGFr) and to report on clinical efficacy. HuMax-EGFR was administered up to five times in doss up to 8 mg/kg. Among 28 patients entered, 7 discontinued after single-dose period, leaving 21 to enter the multiple-dose period. Additionally 5 patients discontinued, leaving 16 (57%) to complete the study. Three patients died in the treatment period. Most frequently reported adverse event was rash. All but one event were CTC grade 1 or 2 and a dose-dependent relationship was indicated. Duration of skin reactions varied from few days to 2 months. No DLTs were observed and MTD was not reached. In the two highest dose groups, 7 of 11 patients obtained a PR or SD and 9 patients obtained metabolic PR or SD. HuMax-EGFr can be safely administered in doses up to 8 mg/kg, and preliminary data on tumour response are encouraging.

An open-label single-arm, phase II trial of zalutumumab, a human monoclonalanti-EGFR antibody, in patients with platinum-refractory squamous cell carcinoma of the head and neck (Saloura et al. 2014). Twenty-three percent of patients had performance status (PS) 2 and 74% had distant metastases. Median OS was 5.3 months (95% CI [4.1, 7.1]), and median PFS was 2.1 months (95% CI [2.0, 2.6]). Subgroup analysis by ECOG PS revealed median OS of 6.3 months for PS = 0–1 and 2.5 months for PS = 2. Objective response rate was 5.7%, and disease control rate was 39.8%. Grade 3–4 adverse events related to zalutumumab were observed in 19% of patients and included skin rash (5%), hypomagnesemia (4%) and pneumonitis (1%). The frequency of all-cause grade 3–4 AEs was 62% and included infections (14%), gastrointestinal disorders (12%) and hypokalemia (6%). Two deaths were deemed related to zalutumumab.

EGFR MoAb - Panitumumab

Cisplatin and fluorouracil with or without panitumumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck (SPECTRUM): an open-label phase 3 randomised trial (Vermorken et al. 2013a). Although the addition of panitumumab to chemotherapy did not improve overall survival in an unselected population of patients with recurrent or metastatic SCCHN, it improved progression-free survival and had an acceptable toxicity profile. p16 status could be a prognostic and predictive marker in patients treated with panitumumab and chemotherapy. Prospective assessment will be necessary to validate our biomarker findings.

Chemoradiotherapy with or without panitumumab in patients with unresected, locally advanced squamous-cell carcinoma of the head and neck (CONCERT-1): a randomised, controlled, open-label phase 2 trial (Mesía et al. 2015).

Between Oct 26, 2007, and March 26, 2009, 153 patients were enrolled and 150 received treatment (63 in the chemoradiotherapy group and 87 in the panitumumab plus chemoradiotherapy group). Local-regional control at 2 years was 68% (95% CI 54–78) in the chemoradiotherapy group and 61% (50–71) in the panitumumab plus chemoradiotherapy group. The most frequent grade 3–4 adverse events were dysphagia (17 [27%] of 63 patients in the chemoradiotherapy group vs. 35 [40%] of 87 in the panitumumab plus chemoradiotherapy group), mucosal inflammation (15 [24%] vs. 48 [55%]), and radiation skin injury (eight [13%] vs. 27 [31%]). Serious adverse events were reported in 20 (32%) of 63 patients in the chemoradiotherapy group and in 37 (43%) of 87 patients in the panitumumab plus chemoradiotherapy group.

Panitumumab plus radiotherapy versus chemoradiotherapy in patients with unresected, locally advanced squamous-cell carcinoma of the head and neck (CONCERT-2): a randomised, controlled, open-label phase 2 trial (Giralt et al. 2015). 152 patients were enrolled, and 151 received treatment (61 in the chemoradiotherapy group and 90 in the radiotherapy plus panitumumab group). Local-regional control at 2 years was 61% (95% CI 47–72) in the chemoradiotherapy group and 51% (40–62) in the radiotherapy plus panitumumab group. The most frequent grade 3–4 adverse events were mucosal inflammation (25 [40%] of 62 patients in the chemoradiotherapy group vs. 37 [42%] of 89 patients in the radiotherapy plus panitumumab group), dysphagia (20 [32%] vs. 36 [40%]), and radiation skin injury (seven [11%] vs. 21 [24%]). Serious adverse events were reported in 25 (40%) of 62 patients in the chemoradiotherapy group and in 30 (34%) of 89 patients in the radiotherapy plus panitumumab group. Panitumumab cannot replace cisplatin in the combined treatment with radiotherapy for unresected stage III-IVb squamous-cell carcinoma of the head and neck, and the role of EGFR inhibition in locally advanced squamous-cell carcinoma of the head and neck needs to be reassessed.

10.4.2 Synchronus VEGF and EGFR blocking agents

Bevacizumab

Reported an open-label, non-randomized single-institution prospective trial of synchronous VEGF and EGFR blockade using (1) bevacizumab (10 mg/kg) every two weeks, (2) 100 mg erlotinib daily or (3) both agents, with concurrent chemoradiation (Cisplatin) in non metastatic, locally advanced head and neck cancer (Yoo et al. 2012). Twenty-nine patients enrolled on study, with 27 completing therapy. The primary objective of the study included safety and efficacy endpoints. Clinical CR after CRT was 96% [95% confidence interval (CI), 82–100%]. Median follow-up was 46 months in survivors, with 3-year locoregional

control and distant metastasis-free survival rates of 85 and 93%. Three-year estimated progression-free survival, disease-specific survival, and overall survival rates were 82, 89, and 86%, respectively. Common grade III toxicities were mucositis (n = 14; 50%), dysphagia (n = 8; 29%), dehydration (n = 7), osteoradionecrosis (n = 3; 11%), and soft tissue necrosis (n = 2). There were no treatment related deaths. Overall, treatment was feasible, with one patient not completing protocol-specified therapy. The trial confirmed the utility of dual VEGF/EGFR inhibition can be integrated with chemoradiotherapy in locally advanced HNC with efficacy.

Combined modality treatment with chemotherapy, radiation therapy, bevacizumab, and erlotinib in patients with locally advanced squamous carcinoma of the head and neck: a phase II trial of the Sarah Cannon oncology research consortium (Hainsworth et al. 2011). After a median follow up of 32 months, the estimated 3-year progression-free and overall survival rates are 71 and 82%, respectively. Sixty-five percent of patients had major responses after induction therapy; after completion of therapy, 95% of patients had either partial or complete response radiographically. As expected, grade 3/4 mucosal toxicity occurred frequently (88%) during combined modality; no unexpected toxicity resulted from the addition of bevacizumab and erlotinib.

Cetuximab and bevacizumab: preclinical data and phase II trial in recurrent or metastatic squamous cell carcinoma of the head and neck (Argiris et al. 2013a). Cetuximab plus bevacizumab enhanced growth inhibition both in vitro and in vivo, and resulted in potent reduction in tumor vascularization. In the clinical trial, 46 eligible patients were enrolled. The objective response rate was 16% and the disease control rate 73%. The median progression-free survival and overall survival were 2.8 and 7.5 months, respectively. Grade 3–4 adverse events were expected and occurred in less than 10% of patients. transforming growth factor alpha, placenta-derived growth factor, EGFR, VEGFR2 increased and VEGF decreased after treatment but did not correlate with treatment efficacy.

Phase I study of bevacizumab added to fluorouracil- and hydroxyurea-based concomitant chemoradiotherapy for poor-prognosis head and neck cancer (Seiwert et al. 2008). Forty-three patients were treated. DLT was reached at level 3 (bevacizumab 5 mg/kg, FU 800 mg/m(2), HU 1000 mg) with two grade 3 transaminase elevations and one grade 4 neutropenia, attributed to the combination of chemotherapy with bevacizumab. For level 4, chemotherapy doses were reduced (FU 600 mg/(2), HU 500 mg), and bevacizumab escalation continued to 10 mg/kg. Treatment of six assessable patients resulted in one venous thrombosis; this dose level was expanded to 26 patients. Late complications included five patients with fistula formation (11.6%) and four with ulceration/tissue necrosis (9.3%). Serious toxicities (hemorrhage/thrombosis/death) were comparable to prior reirradiation reports. Median overall survival for reirradiated patients with recurrent, non-metastatic disease was 10.3 months [95% CI, 5.6–13.5]; 2-year cumulative incidence of death resulting from disease was 51.7% (95% CI, 31.7–68.5).

Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study (Cohen et al. 2009). In the phase I section of the trial, ten patients were enrolled in three successive cohorts with no dose-limiting toxic effects noted. 46 patients were enrolled in the phase II section of the trial (including three patients from the phase I section) on the highest dose of bevacizumab (15 mg/kg every 3 weeks). Two additional patients were accrued beyond the protocol-stipulated 46, leaving a total of 48 patients for the phase II assessment. The most common toxic effects of any grade were rash and diarrhoea (41 and 16 of 48 patients, respectively). Three patients had serious bleeding events of grade 3 or higher. Seven patients had a response, with four showing a complete response allowing rejection of the null hypothesis. Median time of overall survival and progression-free survival (PFS) were 7.1 months (95% CI 5.7–9.0) and 4.1 months (2.8–4.4), respectively. Higher ratios of tumour-cell phosphorylated VEGF receptor-2 (pVEGFR2) over total VEGFR2 and endothelial-cell pEGFR over total EGFR in pretreatment biopsies were associated with complete response (0.704 vs. 0.386, $p = 0.036$ and 0.949 vs. 0.332, $p = 0.036$, respectively) and tumour shrinkage ($p = 0.007$ and $p = 0.008$, respectively) in a subset of 11 patients with available tissue.

10.4.3 Sym 004 (anti EGFR antibody mixture)

A proof of concept trial of the anti-EGFR antibody mixture Sym004 in patients with squamous cell carcinoma of the head and neck (Machiels et al. 2015b). Among the 26 patients treated with Sym004, the proportion of patients alive without disease progression at 6 months was 12% (95% CI 1–39%). The median duration of progression-free survival was 82 days (95% CI 41–140 days). Of 19 patients evaluable for response, eight showed a decrease in the sum of the largest diameter in their target lesions (median 11%; range 7–27%). The best overall response was stable disease in 13 patients (50%). Paired biopsies showed a significant down-regulation of EGFR in both skin and tumors following exposure to Sym004. All patients had EGFR-related adverse events, including grade 3 skin toxicities and grade ≥ 3 hypomagnesemia reported in 13 (50%) and 10 (38%) of 26 patients, respectively. One event fulfilling the protocol-defined criteria for infusion-related reactions (grade 2) was reported. No anti-drug antibodies were detected.

10.4.4 EGFR TKI

Gefitinib

Single-arm phase II study of multiagent concurrent chemoradiotherapy and gefitinib in locoregionally advanced squamous cell carcinoma of the head and neck (Rodriguez et al. 2012). Sixty patients were enrolled in the study; 80% had

stage IV disease and 68% had oropharyngeal primary tumors. The full course of gefitinib was not tolerated by 42%; there were 5 treatment-related deaths (8%). With a median follow-up of 54 months, 2- and 3-year overall survival estimates were 80 and 71%, respectively. Projected distant metastatic control at 2 and 3 years was 88%. When compared with our historical cohort, acute toxicities including renal dysfunction and unplanned rehospitalization were worse in the study patients. Projected outcome estimates did not differ between the 2 cohorts.

Gefitinib plus cisplatin and radiotherapy in previously untreated head and neck squamous cell carcinoma: a phase II, randomized, double-blind, placebo-controlled study (Gregoire et al. 2011). Gefitinib (250 and 500 mg/day) did not improve 2-year LDCR compared with placebo either when given concomitantly with chemoradiotherapy (32.7% vs. 33.6%, respectively; OR 0.921, 95% CI 0.508, 1.670 [1-sided $p = 0.607$]) or as maintenance therapy (28.8% vs. 37.4%, respectively; OR 0.684, 95% CI 0.377, 1.241 [1-sided $p = 0.894$]). Secondary efficacy outcomes were broadly consistent with the 2-year LDCR results. In both doses, gefitinib was well-tolerated and did not adversely affect the safety and tolerability of concomitant chemoradiotherapy.

Phase II study of gefitinib adaptive dose escalation to skin toxicity in recurrent or metastatic squamous cell carcinoma of the head and neck (Perez et al. 2012). Forty four patients were enrolled. Only twenty-three (52%) experienced skin rash grade ≥ 2. Of 44 patients, partial responses were noted in 3 (7%), stable disease in 8 (18%) and progressive disease in 33 patients. Median progression-free survival was 1.9 months (95% CI 1.6–2.2) and median overall survival was 5.1 months (95% CI 2.4–7.8). Grade of skin rash was not associated with response rate ($p = 0.169$) nor tumor control rate ($p = 0.284$); however, higher gefitinib trough levels were associated with disease control. Of the 11 tissue samples analyzed for EGFR gene copy by FISH, 7 were EGFR FISH positive, but this was not associated with improved tumor control or survival.

Molecular and clinical responses in a pilot study of gefitinib with paclitaxel and radiation in locally advanced head-and-neck cancer (Van Waes et al. 2010). Ten patients were treated. The MTD of this combination was GEF 250 mg/d with PAC 36 mg/m(2) intravenously weekly $\times$ 6 with concurrent RT. Grade 3/4 toxicities included prolonged (>8 weeks) stomatitis (7 patients), infection (2 patients), and interstitial pneumonitis (1 patient). There were five complete responses (CR) and two partial responses (PR). Of 7 patients undergoing serial biopsies, only 1 patient demonstrated a reduction in phosphorylated EGFR, decreased downstream signaling, and reduced cellular proliferation after initiating GEF.

Phase III randomized, placebo-controlled trial of docetaxel with or without gefitinib in recurrent or metastatic head and neck cancer (Argiris et al. 2013b). Two hundred seventy patients were enrolled before the study was closed early at interim analysis (arm A, n = 136; arm B, n = 134). Median overall survival was 6.0 months in arm A versus 7.3 months in arm B (hazard ratio, 0.93; 95% CI, 0.72–1.21; $P = 0.60$). An unplanned subset analysis showed that gefitinib improved

survival in patients younger than 65 years (median 7.6 vs. 5.2 months; $P = 0.04$). Also, there was a trend for improved survival in patients with c-MET wild-type (5.7 vs. 3.6 months; $P = 0.09$) regardless of treatment. Grade 3/4 toxicities were comparable between the two arms except that grade 3/4 diarrhoea was more common with docetaxel/gefitinib. Of 18 eligible patients who received gefitinib after disease progression in arm A, one patient had a partial response.

Neoadjuvant chemotherapy/gefitinib followed by concurrent chemotherapy/radiation therapy/gefitinib for patients with locally advanced squamous carcinoma of the head and neck (Hainsworth et al. 2009). Sixty-two patients (53% with stage IV disease) received protocol treatment, and 50 patients (81%) were able to complete the regimen. The addition of gefitinib increased the incidence of grade 3/4 mucositis (27%) and diarrhea (16%) during induction therapy but did not appear to add substantially to toxicity during concurrent chemoradiation. The estimated 3-year progression-free and overall survival rates for the entire group were 41 and 54%, respectively.

Phase I trial of gefitinib incombination with radiation or chemoradiation for patients with locally advanced squamous cell head and neck cancer (Chen et al. 2007). Twenty-three patients were enrolled and assessable for toxicity. No dose-limiting toxicities (DLTs) were observed in patients treated in cohort I at either 250 or 500 mg of gefitinib daily with concomitant boost RT to 72 Gy. In patients receiving chemoradiotherapy and gefitinib (cohort II), DLTs included one grade 4 diarrhea and one grade 4 neutropenic fever. Fifteen patients started maintenance gefitinib, and eight (53%) experienced grade 1–2 acne-like skin rash and diarrhea, but no grade 3 or 4 toxicity occurred.

Contrasted outcomes to gefitinib on tumoral IGF1R expression in head and neck cancer patients receiving postoperative chemoradiation (GORTEC trial 2004-02) (Thariat et al. 2012). Seventy-nine patients were included in this biomarker study, whereas 27 did not meet prerequisites for randomization between gefitinib and placebo. Two-year disease-free survival (DFS) rate was 65.0% and did not differ between randomized patients treated with gefitinib or placebo ($P = 0.85$). The similarity of DFS curves between nonrandomized patients (n = 27), randomized patients without gefitinib (n = 27), and randomized patients receiving gefitinib (n = 25), and similar histoclinical parameter distributions for all groups, allowed us to conduct statistical analyses on the entire population. On multivariate analysis, elevated expression of PAK1 by Western blotting, CD31 and membranous insulin-like growth factor 1 receptor (IGF1R) both by immunohistochemistry was significantly associated with shorter DFS. There was a significant interaction between IGF1R and gefitinib. Gefitinib abolished the prognostic discriminative power of high IGF1R expression; patients with elevated IGF1R expression benefited from gefitinib whereas those with low IGF1R fared worse.

10.4.5 Dual TKI (EGFR/HER2)

Lapatinib

Postoperative Adjuvant Lapatinib and Concurrent Chemoradiotherapy Followed by Maintenance Lapatinib Monotherapy in High-Risk Patients With Resected Squamous Cell Carcinoma of the Head and Neck: A Phase III, Randomized, Double-Blind, Placebo-Controlled Study (Harrington et al. 2015). Six hundred eighty-eight patients were enrolled (lapatinib, n = 346; placebo, n = 342). With a median follow-up time of 35.3 months, the study ended early because of the apparent plateauing of disease-free survival (DFS) events. Median DFS assessed by an independent review committee was 53.6 months and not reached for lapatinib and placebo, respectively (hazard ratio, 1.10; 95% CI, 0.85–1.43). Investigator-assessed results confirmed the independent review committee assessment. No significant differences in DFS by human papillomavirus status or overall survival were observed between treatment arms. Similar numbers of patients in both treatment arms experienced adverse events (AEs), with more patients in the lapatinib arm than the placebo arm experiencing serious AEs (48% vs. 40%, respectively). The most commonly observed treatment-related AEs were diarrhea and rash, both predominantly in the lapatinib arm.

A phase II study of lapatinib inrecurrent/metastatic squamous cell carcinoma of the head and neck (de Souza et al. 2012). Forty-five patients were enrolled, 27 in arm A and 18 in arm B. Diarrhea was the most frequent toxicity occurring in 49% of patients. Seven patients experienced related grade 3 toxicity (3 fatigue, 2 hyponatremia, 1 vomiting, and 1 diarrhea). In an intent-to-treat analysis, no complete or partial responses were observed, and stable disease was the best response observed in 41% of arm A (median duration, 50 days, range, 34–159) and 17% of arm B subjects (median, 163 days, range, 135–195). Median PFS was 52 days in both arms. Median OS was 288 (95% CI, 62–374) and 155 (95% CI, 75–242) days for arms A and B, respectively. Correlative analyses revealed an absence of EGFR inhibition in tumor tissue.

Effects of lapatinib monotherapy: results of a randomised phase II study in therapy-naive patients with locally advanced squamous cell carcinoma of the head and neck (Del Campo et al. 2011). Versus placebo, lapatinib monotherapy did not significantly increase apoptosis detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling or caspase-3 assays. A statistically significant decrease in proliferation using Ki67 assay was observed ($P = 0.030$). In a subset of 40 patients that received 4 weeks of lapatinib or placebo, objective response rate (ORR) was 17% (n = 4/24) versus 0% (n = 0/16). In the lapatinib single-agent responders, all had EGFR overexpression, 50% had EGFR amplification, and 50% had HER2 expression by immunohistochemistry (including one patient with HER2 amplification). However, these patients showed variable modulation of apoptosis, proliferation, and phosphorylated EGFR on drug treatment. Following CRT, there was a statistically non-significant difference in ORR between lapatinib (70%) and placebo (53%). There was no clear

correlation between changes in apoptosis or proliferation and response to chemoradiation. Mucosal inflammation, asthenia, odynophagia, and dysphagia were the most commonly reported adverse events with lapatinib.

10.4.6 VEGFR TKI

Afatinib

Afatinib versus methotrexate as second-line treatment in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck progressing on or after platinum-based therapy (LUX-Head & Neck 1): an open-label, randomised phase 3 trial (Machiels et al. 2015a). Between Jan 10, 2012, and Dec 12, 2013, we enrolled 483 patients and randomly assigned 322 to afatinib and 161 to methotrexate. After a median follow-up of 6.7 months (IQR 3.1–9.0), progression-free survival was longer in the afatinib group than in the methotrexate group (median 2.6 months [95% CI 2.0–2.7] for the afatinib group vs. 1.7 months [1.5–2.4] for the methotrexate group; hazard ratio [HR] 0.80 [95% CI 0.65–0.98], $p = 0.030$). The most frequent grade 3 or 4 drug-related adverse events were rash or acne (31 [10%] of 320 patients in the afatinib group vs. none of 160 patients in the methotrexate group), diarrhoea (30 [9%] vs. three [2%]), stomatitis (20 [6%] vs. 13 [8%]), fatigue (18 [6%] vs. five [3%]), and neutropenia (1 [<1%] vs. 11 [7%]); serious adverse events occurred in 44 (14%) of afatinib-treated patients and 18 (11%) of methotrexate-treated patients.

10.4.7 EGFR TKI

Erlotinib

Phase I and pharmacokinetic study of erlotinib (OSI-774) in combination with docetaxel in squamous cell carcinoma of the head and neck (SSCHN) (Kraut et al. 2011). Ninety-five courses were successfully given (median 3, range 1–6). The most frequent side effects were diarrhoea, fatigue, skin rash, anemia, and hypoalbuminemia. Dose de-escalation for both erlotinib and docetaxel was due to skin rash, neutropenia and/or severe infection with docetaxel to 25 mg/m^2 and erlotinib to starting dose of 50 mg and re-escalation of docetaxel to 35 mg/m^2. Responses were observed in 4/26 evaluable patients (100 mg erlotinib). In 24 patients, the mean Cmax and AUC erlotinib values increased with dose and following cumulative dosing (days 7 and 8 vs. day 1, $p < 0.05$). The CL/F ($\sim$7 L/h), V/F ($\sim$140 L), and t1/2 ($\sim$20 h) for erlotinib were similar to the reported. The mean AUC ratio of metabolite OSI-420 to erlotinib following repetitive dosing at 100 mg (+ or − docetaxel) showed a $\sim$50% increase ($p < 0.02$), possibly suggesting self-enzyme induction. Population pharmacokinetic studies showed no significant covariate affecting erlotinib pharmacokinetics.

Cisplatin and radiotherapy with or without erlotinib in locally advanced squamous cell carcinoma of the head and neck: a randomized phase II trial (Martins et al. 2013). Between December 2006 and October 2011, 204 patients were randomly assigned. Arms were well balanced for all patient characteristics including p16, with the exception of more women on arm A. Patients on arm B had more rash, but treatment arms did not differ regarding rates of other grade 3 or 4 toxicities. Arm A had a CRR of 40% and arm B had a CRR of 52% ($P = 0.08$) when evaluated by central review. With a median follow-up time of 26 months and 54 progression events, there was no difference in PFS (hazard ratio, 0.9; $P = 0.71$).

Erlotinib, erlotinib-sulindac versus placebo: a randomized, double-blind, placebo-controlled window trial in operable head and neck cancer (Gross et al. 2014). Ki67 was significantly decreased by erlotinib or erlotinib-sulindac (omnibus comparison, two-sided Kruskal-Wallis, $P = 0.04$). Wilcoxon pairwise contrasts confirmed greater Ki67 effect in both erlotinib groups (erlotinib-sulindac vs. placebo, $P = 0.043$; erlotinib vs. placebo, $P = 0.027$). There was a significant trend in ordering of Ki67 reduction: erlotinib-sulindac > erlotinib > placebo (two-sided exact Jonckheere-Terpstra, $P = 0.0185$). Low baseline pSrc correlated with greater Ki67 reduction (R(2) = 0.312, $P = 0.024$).

10.4.8 Multikinase inhibitors (MKI)

Sunitinib

Phase II study of sunitinib malate in head and neck squamous cell carcinoma (Choong et al. 2010). Twenty-two patients were accrued (Cohort A—15 patients, Cohort B—7 patients). Median age in cohort A and B was 56 and 61 years, respectively. Grade 3 hematologic toxicities encountered were lymphopenia (18%), neutropenia (14%) and thrombocytopenia (5%). There was only one incidence of grade 4 hematologic toxicity which was thrombocytopenia. Fatigue and anorexia were the most common non-hematologic toxicities. Grade 3 fatigue occurred in 23% of patients. The only grade 4 non-hematologic toxicity was one incidence of gastrointestinal hemorrhage. Non-fatal hemorrhagic complications occurred in 8 patients: epistaxis (3 patients), pulmonary hemorrhage (2 patients), gastrointestinal hemorrhage (2 patients) and tumor hemorrhage (1 patient). Four patients were not evaluable for tumor response (Cohort A—3 patients, Cohort B—1 patient). One partial response was observed in the entire study. Dose reduction was required in 5 patients (Cohort A—3 patients for grd 3 fatigue, grd 3 mucositis and recurrent grd 3 neutropenia; Cohort B—2 patients for grd 3 fatigue and grd 3 nausea). Median time to progression for cohort A and B were 8.4 and 10.5 weeks, respectively. Median overall survival for cohort A and B was 21 and 19 weeks, respectively.

Phase II study of sunitinib in recurrent or metastatic squamous cell carcinoma of the head and neck: GORTEC 2006-01 (Machiels et al. 2010). A PR was observed in one patient, SD in 18, and PD in 19 (Response Evaluation Criteria in Solid Tumors [RECIST]), resulting in a disease control rate of 50%. Among the

18 patients with SD, there were five unconfirmed PRs and six additional minor responses. A significant decrease in K (trans) was seen in three of the four patients who received DCE-MRI monitoring. Grade 5 head and neck bleeds occurred in four patients. Local complications, including the appearance or worsening of tumor skin ulceration or tumor fistula, were recorded in 15 patients.

10.4.9 VEGFR inhibitors—MKI

Vandetanib

A randomized phase II study of docetaxel with or without vandetanib in recurrent or metastatic squamous cell carcinoma of head and neck (SCCHN) (Limaye et al. 2013). 29 analyzable patients were enrolled, 14 in docetaxel arm and 15 in combined arm. PR was achieved in 1 patient in the docetaxel arm and 2 patients in the combined arm. The objective RR was 7% (1/14) (95% CI 0.2–33.8%) in the single and 13% (2/15) (95% CI 1.6–40.4%) combined arm. The median PFS was 3.21 (95% CI 3.0–22.0) and 9 (95% CI (5.86–18.1) weeks; median OS was 26.8(95% CI 17.7–100.7+) and 24.1 (95% CI, 16.4–171.1+) weeks. Most common adverse events were fatigue, dysphagia, diarrhea or constipation, cytopenias and alopecia.

Phase I study of vandetanib with radiation therapy with or without cisplatin in locally advanced head and neck squamous cell carcinoma (Papadimitrakopoulou et al. 2014). Of 33 treated patients, 30 completed therapy (regimen 1, n = 12; regimen 2, n = 18). MTD in regimen 2 was 100 mg [3 dose limiting toxicities (DLT) at 200 mg], while regimen 1 was stopped due to poor recruitment (one DLT at 200 mg). Most common grade ≥ 3 AEs were dysphagia (30%), stomatitis (33%) and mucosal inflammation (27%). Five patients discontinued vandetanib due to AEs. Conclusions: Vandetanib with chemo RT was feasible. Head Neck, 2014.

10.5 Other Molecular Target Agents

10.5.1 Mammalian Target of Rapamycin (mTOR) Inhibitor

Everolimus

A phase 1 study of everolimus + weekly cisplatin + intensity modulated radiation therapy in head-and-neck cancer (Fury et al. 2013a). Tumor primary sites were oral cavity (4), salivary gland (4), oropharynx (2), nasopharynx (1), scalp (1), and neck node with occult primary (1). In 4 of 4 cases in which resected HNSCC surgical pathology specimens were available for immunohistochemistry, elevated expression of eIF4E was observed in the cancer-free margins. The most common grade ≥ 3 treatment-related adverse event was lymphopenia (92%), and

dose-limiting toxicities (DLTs) were mucositis (n = 2) and failure to thrive (n = 1). With a median follow up of 19.4 months, 2 patients have experienced recurrent disease. The maximum tolerated dose was everolimus 5 mg/day.

A phase 1 study of everolimus plus docetaxel plus cisplatin as induction chemotherapy for patients with locally and/or regionally advanced head and neck cancer (Fury et al. 2013b). Eighteen patients were enrolled (15 men, 3 women), and their median Karnofsky performance status was 90. The most common toxicities were hyperglycemia, low hemoglobin, fatigue, and thrombocytopenia. Dose-limiting toxicities (DLTs) were neutropenic fever (1 event at dose level 2, 2 events at dose level 3), and all patients recovered fully from these DLTs. The maximum tolerated dose was exceeded at dose level 3. The progression-free survival rate at 1 year was 87.5% (95% confidence interval, 56.8–96.7%); and, at 2 years, it was 76.6% (95% confidence interval, 41.2–92.3%). Activating PI3K catalytic subunit α (PIK3CA) gene mutations were identified in 2 human papillomavirus-associated oropharyngeal cancers.

Response-adapted volume de-escalation (RAVD) in locally advanced head and neck cancer (Villaflor et al. 2016). Ninety-four patients were enrolled. Randomization to everolimus was discontinued on interim analysis after 50 patients due to futility. IC response was evaluable in 89 patients. Thirty-seven patients (41.6%) had GR and 52 (58.4%) had NR. There was a trend for improved progression-free ($P = 0.086$) but not overall survival ($P = 0.94$) for GR versus NR. The 2-year PFS and OS were 86.0 and 83.5% for GR and 68.7 and 85.4% for NR, respectively. NR were significantly more likely to undergo G-tube placement during treatment (50.0% GR vs. 73.5% NR, $P = 0.040$) and be G-tube dependent at 6-months follow-up (5.7% GR vs. 32.6% NR, $P = 0.005$).

Temsirolimus

A phase II study of temsirolimus and erlotinib in patients with recurrent and/or metastatic, platinum-refractory head and neck squamous cell carcinoma (Bauman et al. 2013). Twelve patients enrolled; six withdrew within 6 weeks due to toxicity or death, prompting early closure of the trial. Grade ≥ 3 toxicities included fatigue, diarrhea, gastrostomy tube infection, peritonitis, pneumonia, dyspnea, and HN edema. Median PFS was 1.9 months. Median. overall survival was 4.0 months. Six/12 tumors were p16(+), 9/11 lacked measurable PTEN expression, and 1/12 harbored a PIK3CA mutation. On exploratory analysis, high baseline plasma VEGF and interferon-gamma levels marginally associated with tumor progression.

A single-arm multicentre phase II study of temsirolimus in platin- and cetuximab refractory recurrent and/or metastatic squamous cell carcinoma of the head and neck (SCCHN) of the German SCCHN Group (AIO) (Grünwald et al. 2015). A total of 40 patients were eligible. The PFR at 12 weeks was 40% (95% CI 25.0–54.6). The median PFS and OS were 56 days (95% CI 36–113 days) and 152 days (76–256 days), respectively. In 33 assessable patients, disease stabilization occurred in 57.6%, with tumor shrinkage in 13 patients (39.4%). Overall, the treatment was well tolerated. Fatigue (47.5%), anemia (25.0%), nausea (20.0%), and pneumonia (20.0%) were the most common adverse events. Neither PIK3CA mutations, nor HPV status were predictive for success with temsirolimus treatment. No mutations were found for KRAS or BRAF.

10.5.2 Proteasome inhibitor

Bortezomib

Nuclear factor-kappa B pathway and response in a phase II trial of bortezomib and docetaxel in patients with recurrent and/or metastatic head and neck squamous cell carcinoma (Chung et al. 2010). Twenty-one of 25 enrolled patients were assessable for response; one partial response (PR, 5%), 10 stable disease (SD, 48%) and 10 progressive disease (PD, 48%). Patients with PR/SD had significantly longer survival compared with patients with PD and the regimen was well tolerated. Only one of 20 tumors was positive for HPV. Patients with PD had higher expression of NF-kappaB and epidermal growth factor receptor-associated genes in their tumors by gene expression analysis.

Phase II 2-arm trial of the proteasome inhibitor, PS-341 (bortezomib) in combination with irinotecan or PS-341 alone followed by the addition of irinotecan at time of progression in patients with locally recurrent or metastatic squamous cell carcinoma of the head and neck (E1304): a trial of the Eastern Cooperative Oncology Group (Gilbert et al. 2013). The response rate of bortezomib and irinotecan was 13%. One patient had a partial response to bortezomib alone (response rate 3%). No responses were seen in patients with addition of irinotecan at time of progression on bortezomib.

10.5.3 MET/VEGFR inhibitor

Foretinib: GSK1363089 (XL880)

Phase II trial of single-agent foretinib (GSK1363089) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck (Seiwert et al. 2013). Fourteen patients were enrolled. The study did not meet criteria for continuing to the second stage. A maximum of 30 cycles were administered (median = 4.0). Fifty percent of patients (7/14) showed stable disease (SD), 43% of patients (6/14) experienced tumor shrinkage and two patients had prolonged disease stabilization for $\geq$ 13 months. The most common adverse events were fatigue, constipation and hypertension, which were manageable with additional medication or adjustments to the dosing schedule.

10.5.4 Adenovirus gene therapy

INGN 201 (Ad5CMV-p53)

A phase 2 trial of surgery with perioperative INGN 201 (Ad5CMV-p53) gene therapy followed by chemoradiotherapy for advanced, resectable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx: report of the Southwest Oncology Group (Yoo et al. 2009). All 13 patients

received surgery and perioperative INGN 201 injections in the primary tumor bed and the ipsilateral neck. In addition, 3 patients received injections in the contralateral neck. Three patients did not receive chemoradiotherapy. One patient had a grade 2 fistula of the oral cavity. Of the 10 patients with evaluable data, 2 experienced grade 4 adverse events, 1 owing to hypokalemia, hyponatremia, vomiting, leukopenia, and neutropenia and 1 owing to increased aspartate aminotransferase and alanine aminotransferase levels. Seven other patients experienced grade 3 adverse events. The estimate of 1-year progression-free survival is 92%.

10.5.5 Aurora A kinase inhibitor

Alisertib (MLN8237)

Safety and activity of alisertib, an investigational aurora kinase A inhibitor, in patients with breast cancer, small-cell lung cancer, non-small-cell lung cancer, head and neck squamous-cell carcinoma, and gastro-oesophageal adenocarcinoma: a five-armphase 2 study (Melichar et al. 2015). 249 patients had been treated, 53 with breast cancer, 60 with small-cell lung cancer, 26 with non-small-cell lung cancer, 55 with head and neck squamous-cell carcinoma, and 55 with gastro-oesophageal adenocarcinoma. Among response-assessable patients, an objective response was noted in nine (18%, 95% CI 9–32) of 49 women with breast cancer, ten (21%, 10–35) of 48 participants with small-cell lung cancer, one (4%, 0–22) of 23 patients with non-small-cell lung cancer, four (9%, 2–21) of 45 people with head and neck squamous-cell carcinoma, and four (9%, 2–20) of 47 individuals with gastro-oesophageal adenocarcinoma; all were partial responses. Adverse events were similar across tumour types. The most frequent drug-related grade 3–4 adverse events included neutropenia (n = 107 [43%]), leukopenia (53 [21%]), and anaemia (26 [10%]). Serious drug-related adverse events were reported in 108 (43%) patients.

10.5.6 Src/Abl kinase inhibitor

Saracatinib (AZD0530)

Phase II study of saracatinib (AZD0530) for patients with recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) (Fury et al. 2011). Nine patients were enrolled. All patients had received prior radiotherapy and six patients had received prior chemotherapy for recurrent or metastatic disease. The most common adverse event was fatigue. Eight patients had progression of disease by response evaluation criteria in solid tumors (RECIST) within the first eight-week cycle and one patient was removed from the study after 11 days due to clinical decline with stable disease according to the RECIST criteria. Median overall survival was six months. The study was closed early due to lack of efficacy according to the early stopping rule.

10.6 Outcomes

10.6.1 Responses

The most trials using molecular target agents have been carried out in patients with locally advanced, recurrent or metastatic squamous-cell carcinoma of the head and neck as second-line treatments after failure of platinum-based chemotherapy or first-line treatments in combination with platinum-based chemotherapy. Among 181 trials registered in ClinicalTrials.gov, 55 studies (30%) with primary objective of "response to therapy" were selected for evaluation. The therapeutic effects of targeted agents tried in various trials vary with response rates (RR) between 7 and 96%.

Single agents (monotherapy)

With regards to the trials for single agents, 14 studies for LRC, PFR, TR and ORR were selected, and 9 out of 14 trials that have published were selected for the evaluation. Among those, cetuximab, lapatinib, gefitinib, sorafenib, sunitinib, ZD1839, GSK1363089, temsirolimus, P276-00, rapamycin, dalantercept, BKM120 and AZD0530 were included, and some of the agents showed clinical activities as monotherapy in SCCHN. In a open-label, multi-institution, phase II study (Perez et al. 2012) evaluated the activity of gefitinib at individually escalated doses up to 750 mg to achieve the skin toxicity grade P2, and achieved 7% (3 out of 44) partial response (NCT00519077).

There are few reports, however, to support increased performance in terms of responses. Out of 181 trials registered in ClinicalTrials.gov, 39 (22%) were listed as cetuximab trials and of these 23 studies (59%) were RCTs. Among those 8 (35%) studies were published, and there is one study with primary objective for loco-regional control in patients with recurrent or metastatic SCCHN (Bonner et al. 2006) (NCT00004227). In this phase III study, the median duration of loco-regional control was 24.4 months among patients treated with cetuximab plus radiotherapy and 14.9 months among those given radiotherapy alone (hazard ratio for loco-regional progression or death, 0.68; $P = 0.005$). On the other hand, a phase II randomized trial have been conducted to directly compare RT with concomitant cisplatin (CDDP) versus concomitant cetuximab (CTX) as first-line treatment of locally advanced SCCHN (NCT01216020) (Magrini et al. 2016). The results indicated that CTX concomitant to RT lowered compliance and increased acute toxicity rates. Efficacy outcomes were similar in both arms.

Treatment of loco-regionally advanced HNSCC with concomitant high-dose radiotherapy plus cetuximab improves loco-regional control and reduces mortality without increasing the common toxic effects associated with radiotherapy to the head and neck (NCT00004227) (Bonner et al. 2006).

Combined therapy

With regards to combined therapies, there are 44 trials with cetuximab (14) followed by gefitinib (8) bevacizumab (4), everolimus (3), panitumumab (3), vandetanib (2), erlotinib (2), bevacizumab/erlotinib (1), lapatinib (2), Bortezonib (2), temsirolimus (1) and INGN 201 (Ad5CMV-p53) (1).

There are 20 trials with combined therapies in a single arm; 9 with cetuximab followed by gefitinib (3) and erlotinib (3). 64%(28/44) trials included platinum-based therapies.

Two trials with cisplatin, bevacizumab, erlotinib with RT (NCT00140556) (Yoo et al. 2012) and cetuximab, paclitaxel, carboplatin with RT (NCT00343083) (Suntharalingam et al. 2012) have achieved 3-year LRC at 85 and 72%, respectively. Platinum-based regimens have been used for cetuximab, gefitinib, lapatinib, bevacizumab, panitumumab, vandetanib, everolimus, INGN 202 (Table 10.3). On the other hand, afatinib, zalutumumab, Sym004 (anti-EGFR antibody mixture), sunitinib, foretinib and alisertib have been used as a single agent. Among the platinum-based regimens in published studies, the most common targeted agents are cetuximab (14) followed by bevacizumab (5), lapatinib (3) and zalutumumab (3). For cetuximab, 33 trials have been registered (Table 10.3). Among those 19 trials (58%) have not yet provided publications. Two phase II non-randomised trials in cetuximab as the first-line treatments have been carried out in single arm with cisplatin/carboplatin and 5-FU in Japan and China. (NCT00971932, NCT01177956) (Guo et al. 2015; Yoshino et al. 2013), and the best overall response rates were 36.4% (95%CI; 20.4–54.9) and 54.4% (95%CI; 41.9–66.5) respectively.

RTOG0522 (NCT00265941) (Ang et al. 2014), one of the largest phase III studies with 891 analyzed patients, with stage III to IV HNSCC was a randomized trial of concurrent accelerated radiation plus cisplatin with or without cetuximab. The study status is ongoing but not recruiting. The patients were treated with either 70 Gy or 72 Gy in different fractions over 6 weeks and CDDP (100 mg/m^2) on days 1 and 22 of radiotherapy with the cetuximab (400 mg/m^2), the week before radiotherapy and then 250 mg/m^2 per week during radiotherapy. Of the analyzed patients, 630 were alive at analysis (median follow-up, 3.8 years). No differences were found between cisplatin-radiation alone versus cetuximab plus cisplatin-radiation in 30-day mortality (1.8% vs. 2.0%, respectively; $P = 0.81$), 3-year PFS (61.2% vs. 58.9%, respectively; $P = 0.76$) and 3-year OS (72.9% vs. 75.8%, respectively; $P = 0.32$). Patients with p16-positive oropharyngeal carcinoma (OPC), compared with patients with p16-negative OPC, had better 3-year probability of PFS (72.8% vs. 49.2%, respectively; $P < 0.001$) and OS (85.6% vs. 60.1%, respectively; $P < 0.001$), but EGFR expression did not distinguish outcome.

A systematic review and meta-analysis of published studies for concomitant platinum-based chemotherapy or cetuximab with RT for locally advanced HNSCC concluded that platinum-based combinations of radiotherapy (RT) plus chemotherapy (CTRT) with cisplatin is associated with a better OS and PFS compared to RT plus cetuximab (RT+CET), and this is probably attributed to improved loco-regional disease control (Petrelli et al. 2014).

As compared with platinum-based chemotherapy plus fluorouracil alone, cetuximab plus platinum–fluorouracil chemotherapy improved overall survival when given as first-line treatment in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck as applied in the EXTREME trial

(NCT00122460) (Vermorken et al. 2008). Adding cetuximab to platinum-based chemotherapy with fluorouracil significantly prolonged the median overall survival from 7.4 months in the chemotherapy-alone group to 10.1 months in the group that received chemotherapy plus cetuximab (hazard ratio for death, 0.80; 95% CI; 0.64–0.99; $P = 0.04$). The addition of cetuximab prolonged the median progression-free survival time from 3.3 to 5.6 months (hazard ratio for progression, 0.54; $P < 0.001$) and increased the response rate from 20% to 36% ($P < 0.001$) (Vermorken et al. 2008). Among 16 published studies in chemotherapy plus cetuximab, only 4 trials were randomized phase II/III settings (NCT00122460 (Vermorken et al. 2008), NCT00265941 (Ang et al. 2014), NCT00705016 (Vermorken et al. 2014), NCT01081041 (Soulières et al. 2016)). Most of the study results shown limited improvement in the OS, DFS, EFS and PFS.

10.6.2 Survival

Among 182 trials registered in ClinicalTrials.gov, 49 studies that have primary objective of survival were selected for evaluation of the survival rates. Among those, 14 trials with primary objective of survival and posted in ClinicalTrials.gov, and were evaluated by us for this review.

Using cetuximub (250 mg/m^2 or 400 mg/m^2 weekly) in resectable or unresectable locally advanced or recurrent/metastatic squamous cell carcinoma of the head and neck, median progression free survival (PFS) was 1.5–6.4 months as single agent or in combination with pemetrexed, carboplatin/cisplatin, folic acid dietary, Vitamin B12 or EMD 1201081(IMO-2055) or 5-FU or carboplatin, paclitaxel, surgery, RT or cisplatin, RT or cisplatin/carboplatin, 5-FU or RT or cilengitide, 5-FU, cisplatin) (NCT00705016, NCT00096174, NCT01040832, NCT01057589) (Vermorken et al. 2013b; Ruzsa et al. 2014; Egloff et al. 2014; Vermorken et al. 2014).

As compared with platinum-based chemotherapy plus fluorouracil alone, cetuximab plus platinum–fluorouracil chemotherapy improved overall survival when given as first-line treatment in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck as applied in the EXTREME trial (NCT00122460) (Vermorken et al. 2008). This study demonstrated a significant benefit of cetuximab with an improvement in median OS from 7.4 months to 10.1 months ($p = 0.04$).

10.6.3 Toxicity and Side Effects of Targeted Therapy

41 studies with primary objective of recording adverse events were selected. Among those, only 6 trials, that have primary objective of toxicities and posted in ClinicalTrials.gov, have been evaluated.

Many serious side effects are noted in reported trials. These include skin rash, mucositis, vomiting and diarrhoea, gastrointestinal disorders, fatigue, dysphagia, infection & sepsis, neutropenia & leukopenia, respiratory and renal failure. Table 10.4 lists these various toxic effects noted against individual agents. Among those, 3 or higher grade adverse events such as skin rash, mucositis, diarrhea, fatigue, dysphagia and neutropenia which are related to lowered compliance are common in most targeted agents, even in the single arm trials. In cetuximab, frequent grade 3 or more toxicities are skin rash (9–45%) and acneiform rash (12–24%), mucositis (23–79%), fatigue (17 or 24%), dysphasia (21 or 29%), neutropenia (8–79%) and leukopenia (21–40%). Thus the combinations of cetuximab have higher rate of adverse events among molecular targeted agents. In combination of the gefitinib, grade 3 or more mucositis is similar to that of cetuximab, but for dysphasia it was higher than cetuximab (39 or 68%), everolimus is the highest in dysphasia (92%) and lymphopenia (67 or 92%) than those of cetuximab.

10.7 Conclusions

The standard therapy for unresected, locally advanced, recurrent or metastatic SCCHN is platinum-based chemotherapy with or without concomitant or sequential radiotherapy. In the recent years following the discovery of molecular pathways in head and neck cancer targeted therapies have been tested as clinical trials. In this review, it is apparent that more than half of clinical trials on this topic are non-randomized (58%) and made of small sample sizes (112 ± 154 cases). It is quite difficult to gather the outcomes for subgroup analysis in patients with oral cavity cancer with high level of evidence.

It is noteworthy that, one large clinical trial demonstrated a significant benefit of cetuximab with an improvement in median OS from 7.4 months to 10.1 months ($p = 0.04$). The addition of cetuximab prolonged the median progression-free survival time from 3.3 to 5.6 months (hazard ratio for progression, 0.54; $P < 0.001$) and increased the response rate from 20% to 36% ($P < 0.001$).

The implication of these results in clinical practice might represent a new treatment paradigm by replacing standard chemotherapy in the first-line setting, which has been widely used in the last few decades. There is no doubt that head and neck cancer patients harbouring positive EGFR mutations have a biologically different entity that requires personalized treatment strategies, including the use of EGFR TKIs. There are questions raised on the concurrent combination of chemotherapy and EGFR TKIs.

Publication bias may influence the way we interpret data reported from various trials. There is strong evidence of an association between significant results and publication; studies that report positive or significant results are more likely to be published and outcomes that are statistically significant have higher odds of being fully reported (Dwan et al. 2008).

There are limitations in collecting secondary data from trial registries as we have done in this chapter. The proportion of published trials that is registered varies considerably as per type of research, per country and per journal in which the research is published (Viergever and Li 2015). Examining the compliance in reporting Anderson et al. (2015) remarked that most highly likely applicable clinical trials (HLACTs) did not report results to ClinicalTrials.gov in a timely fashion during the study period, though industry-funded trials adhered to legal obligations more often than did trials funded by the NIH or other government or academic institutions.

As treatment strategies evolve improved patient selection will be critical to identify those patients who may benefit from intensified treatment. Though less researched anti VGFR in combination with anti EGFR and inhibition of mTOR also appear promising and warranting further trials.

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S. Warnakulasuriya and Z. Khan (eds.), *Squamous Cell Carcinoma*,
DOI 10.1007/978-94-024-1084-6

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