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Barbara Burtness
Erica A. Golemis *Editors*

Molecular Determinants of Head and Neck Cancer

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Molecular Determinants of Head and Neck Cancer

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Contents

1 Overview: The Pathobiology of Head and Neck Cancer 1
Barbara Burtness and Erica A. Golemis

2 Molecular Features and Treatment Modalities of "Classic" Versus Human Papilloma Virus-Associated Head and Neck Cancer 7
Giannis Mountzios and Amanda Psyrri

3 Epidemiology of HPV in Head and Neck Cancer: Variant Strains, Discrete Protein Function 23
Camille C.R. Ragin and Jeffrey C. Liu

4 EGFR Inhibitors as Therapeutic Agents in Head and Neck Cancer 55
Hanqing Liu, Jennifer R. Cracchiolo, Tim N. Beck, Ilya G. Serebriiskii and Erica A. Golemis

5 The Role of HGF/c-MET in Head and Neck Squamous Cell Carcinoma 91
Tanguy Y. Seiwert, Tim N. Beck and Ravi Salgia

6 Insulin-like Growth Factor-1 Receptors in Head and Neck Cancer . . . 113
Steven A. Rosenzweig and Casey O. Holmes

7 The PI3K Signaling Pathway in Head and Neck Squamous Cell Carcinoma 131
Jason D. Howard and Christine H. Chung

8 Jak/STAT Signaling in HNC 163
Bhavana S. Vangara and Jennifer R. Grandis

9 TGFβ Regulates EMT in Head and Neck Cancer 179
Jill M. Neiman and Xiao-Jing Wang

10 The Wnt/β-catenin Signaling Circuitry in Head and Neck Cancer . . . 199
Rogerio M. Castilho and J. Silvio Gutkind

11 Human Papillomavirus (HPV)-Positive Head and Neck Cancer and the Wnt Signaling Pathway . . . 215
Theodoros Rampias and Amanda Psyrri

12 p53 in Head and Neck Cancer . . . 227
Jong-Lyel Roh and Wayne M. Koch

13 DNA Damage Proteins and Response to Therapy in Head and Neck Cancer . . . 259
Ranee Mehra and Ilya G. Serebriiskii

14 Hypoxia and Radioresistance in Head and Neck Cancer . . . 283
Peiwen Kuo and Quynh-Thu Le

15 Sequencing HNC: Emergence of Notch Signaling . . . 303
Curtis R. Pickering, Thomas J. Ow and Jeffrey N. Myers

16 Gene Expression in HNC . . . 325
Michael F. Ochs and Joseph A. Califano

17 Projections: Novel Therapies for HPV-Negative Cancers of the Head and Neck . . . 343
Barbara Burtness

Index . . . 361

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Chapter 1
Overview: The Pathobiology of Head and Neck Cancer

Barbara Burtness and Erica A. Golemis

Abstract Squamous cell cancers arising in the head and neck, from the nasopharynx to the subglottic larynx, are frequently devastating cancers that afflict patients around the world. Early stage cancers are readily cured with surgery or radiation. In contrast, locally advanced or metastatic cancers require morbid multimodality therapy and nonetheless have high recurrence rates. As this book addresses this difficult disease, it has three main goals. First, it seeks to provide an introduction to the etiology and subclasses of squamous cell carcinomas of the head and neck (SCCHNs), in the context of how these differences affect prognosis. Second, it attempts to summarize the current state of understanding of the genetic, epigenetic, and protein expression changes associated with the various classes of SCCHN. Third, it seeks to define potential therapeutic targets for improved management of the disease in the future.

Keywords Head and neck cancer · Human papillomavirus · Epidermal growth factor receptor (EGFR) · DNA repair · Hypoxia · c-MET · Insulin growth factor receptor (IGF1R) · Notch · Genomics

Squamous cell cancers arising in the head and neck, from the nasopharynx to the subglottic larynx, are frequently devastating cancers that afflict patients around the world. Early stage cancers are readily cured with surgery or radiation. In contrast, locally advanced or metastatic cancers require morbid multimodality therapy and nonetheless have high recurrence rates. As this book addresses this difficult disease, it has three main goals. First, it seeks to provide an introduction to the etiology and subclasses of squamous cell carcinomas of the head and neck (SCCHNs), in the context of how these differences affect prognosis. Second, it attempts to summarize the current state of understanding of the genetic, epigenetic, and protein expression

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changes associated with the various classes of SCCHN. Third, it seeks to define potential therapeutic targets for improved management of the disease in the future.

To provide a brief overview, we begin by introducing the changing epidemiology of SCCHN. Historically, habitual exposures such as tobacco, alcohol, and maté have contributed to onset of SCCHN. However, a rising proportion of oropharynx cancers arise from transforming human papillomavirus (HPV) infection [1]. Although these commonly present at a more advanced nodal stage than HPV-negative tumors, HPV-positive tumors are treatment-responsive, with a high cure rate relative to HPV-negative tumors [2]. Chapter 2 (Psyrri) contrasts the molecular features of HPV-positive versus HPV-negative, predominantly exposure-related SCCHN. Complementing this discussion, Chap. 3 (Ragin) presents the epidemiology of the various types of HPV that contribute to SCCHN pathogenesis and assesses the potential for targeting viral oncoproteins.

The differences in etiology, biomolecular signatures, treatment responsiveness, and prognosis seen between HPV-associated and HPV-negative SCCHN result in different research questions for each type of head and neck cancer. For HPV-associated SCCHNs, it will be necessary to identify biomarkers to distinguish between patients with near certainty of cure and those—perhaps most commonly smokers—with HPV-associated cancer but a higher risk of recurrence [3]. Reduced treatment intensity and concomitant reduction of treatment-related morbidity may be achievable for the former; novel therapies must be evaluated for the latter. In contrast, treatment outcomes have not advanced substantially for patients with HPV-negative SCCHN, despite several decades of research into increasingly intense, long duration, multimodality treatments. These patients need treatments that exploit our advancing understanding of the biology of head and neck cancer. Hence, the remainder of the volume reviews in detail protein signaling pathways and targets of potential therapeutic significance.

The monoclonal antibody cetuximab is the first molecularly targeted therapy proven to advance survival in head and neck cancer [4–6]. Cetuximab targets the extracellular domain of the epidermal growth factor receptor (EGFR, also known as ERBB1): The role of EGFR and the other ERBB proteins (ERBB2/HER2, ERBB3, and ERBB4) is reviewed in Chap. 4 (Golemis), along with evidence that upregulation of receptor tyrosine kinases (RTKs) with partially redundant function may provide resistance to cetuximab and more recently developed EGFR-targeting therapies. In particular, abnormal expression and activation of the RTKs c-MET [7] and the insulin-like growth factor receptor-1 receptor (IGF1R) [8] are emerging as relevant to the pathology of SCCHN and may prove to be important therapeutic targets in this disease. C-MET is discussed in Chap. 5 (Seiwert) and IGF1R in Chap. 6 (Rosenzweig).

A common feature of RTKs is their activation of downstream effector pathways that support tumor growth, survival, and resistance to therapy. In the case of SCCHN, some of the most important of the effectors are themselves mutated or otherwise constitutively activated. Chapter 7 (Chung) describes mutational and indirect activation of the PTEN-PI3K-AKT-mTOR [9] signaling axis in SCCHN, and efforts to target proteins in this pathway. Chapter 8 (Grandis) addresses the role of constitutive

Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling [10] observed in a subset of SCCHNs and the challenges involved in developing therapeutics to target these non-catalytic signaling proteins.

For SCCHN as for many other solid tumors, the chances of successful treatment diminish with more advanced stage. Tumor invasion depends in part on epithelial-mesenchymal transition (EMT), which occurs in response to activation of transforming growth factor β (TGFβ) [11], a transmembrane serine-threonine kinase, and its canonical and non-canonical effectors. EMT alters cell shape and expression of differentiation-associated markers, increases motility and invasion, and intriguingly is linked to the acquisition of stem cell-like features that commonly indicate drug resistance. A current assessment of the complex signaling pathways involved in this malignant progression in SCCHN is summarized in Chap. 9 (Wang). Separately, the Wnt/β-catenin signaling pathway [12] provides an independent input into cell differentiation status and can also affect EMT and therapeutic resistance. A growing body of evidence supports the common deregulation of expression of Wnt signaling proteins in SCCHN, with early efforts to evaluate therapeutic agents targeting some signaling intermediates. Chapter 10 (Gutkind) outlines Wnt/β-catenin signaling in detail, emphasizing HPV-negative tumors, while Chap. 11 (Psyrri) evaluates specific relevance of this signaling pathway in HPV-positive SCCHN.

While targeted therapies are a focus of much interest, the mainstay of clinical management of advanced head and neck cancers remains multimodality management incorporating radiation and cytotoxic agents. As these therapies can induce very significant morbidity, it is critical to better understand genetic factors underlying the differential susceptibility of individual tumors to treatment, allowing better stratification of patients for dose deintensification or intensification. TP53 is a major regulator of response to DNA damage in many tumors, including head and neck [13]: Chap. 12 (Koch) summarizes our current understanding of the profile of p53 mutation in the context of SCCHN treatment. Cells typically respond to DNA damage by activating multiple discrete DNA repair complexes that correct double and single strand breaks, DNA crosslinking, and the presence of defective bases [14]. As outlined in Chap. 13 (Mehra), a growing number of studies have determined that some of the proteins that mediate these repair processes are overexpressed in subsets of SCCHN tumors, predicting poor response to therapy and poor prognosis. Chapter 14 (Le) provides a contrasting focus on the issue of resistance to irradiation and the emerging role of hypoxia in this process.

The entire field of cancer biology is being transformed by the application of powerful new technologies that are elucidating the genome and epigenome. Chapter 15 (Myers) summarizes the results of the first massive analyses of SCCHN tumor genomes [15, 16]. These resulted in the first identification of mutations in Notch as contributing to tumor etiology and yielded numerous other insights into the mutational landscape associated with the pathogenesis of head and neck tumors. Chapter 16 (Califano) matches this analysis with in depth examination of the characteristic mRNA and microRNA (miRNA) expression profiles in SCCHN [17, 18]. The final Chap. 17 (Burtness) summarizes phase I–III clinical trials, ongoing in 2013

to study incorporation of new molecularly targeted agents as well as immune checkpoint inhibitors into the management of HPV-negative cancers, with projections for the future.

As of 2014, there is no magic bullet for head and neck cancer. However, as the chapters collected here make clear, the advances in understanding over the past decade have been immense. Within the next few years, we are on a clear course to have rich profiling at the DNA, mRNA, and protein level for a large group of SCCHNs that have been clinically annotated to establish the relationship of this information to disease prognosis. The pipeline of targeted agents is already rich and still expanding, while at the same time the utilization of cytotoxic agents and irradiation becomes more efficiently focused. Going forward, the main challenge will be integrating available and emerging resources to design the most effective clinical trials and to change the natural history of these difficult cancers.

References

1. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29:4294–301.
2. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2011;363:24–35.
3. Gillison ML, Zhang Q, Jordan R, Xiao W, Westra WH, Trotti A, Spencer S, Harris J, Chung CH, Ang KK. Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. J Clin Oncol. 2012;30:2102–11.
4. Burtness B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005;23:8646–54.
5. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359:1116–27.
6. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354:567–78.
7. Seiwert TY, Jagadeeswaran R, Faoro L, Janamanchi V, Nallasura V, El Dinali M, Yala S, Kanteti R, Cohen EE, Lingen MW, Martin L, Krishnaswamy S, Klein-Szanto A, Christensen JG, Vokes EE, Salgia R. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. Cancer Res. 2009;69:3021–31.
8. Barnes CJ, Ohshiro K, Rayala SK, El-Naggar AK, Kumar R. Insulin-like growth factor receptor as a therapeutic target in head and neck cancer. Clin Cancer Res. 2007;13:4291–9.
9. Bauman JE, Arias-Pulido H, Lee SJ, Fekrazad MH, Ozawa H, Fertig E, Howard J, Bishop J, Wang H, Olson GT, Spafford MJ, Jones DV, Chung CH. A phase II study of temsirolimus and erlotinib in patients with recurrent and/or metastatic, platinum-refractory head and neck squamous cell carcinoma. Oral Oncol. 2013;49:461–7.

10. Leeman RJ, Lui VW, Grandis JR. STAT3 as a therapeutic target in head and neck cancer. Expert Opin Biol Ther. 2006;6:231–41.
11. White RA, Malkoski SP, Wang XJ. TGFbeta signaling in head and neck squamous cell carcinoma. Oncogene. 2010;29:5437–46.
12. Rhee CS, Sen M, Lu D, Wu C, Leoni L, Rubin J, Corr M, Carson DA. Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. Oncogene. 2002;21:6598–605.
13. Skinner HD, Sandulache VC, Ow TJ, Meyn RE, Yordy JS, Beadle BM, Fitzgerald AL, Giri U, Ang KK, Myers JN. TP53 disruptive mutations lead to head and neck cancer treatment failure through inhibition of radiation-induced senescence. Clin Cancer Res. 2012;18:290–300.
14. Iyama T, Wilson DM. 3rd DNA repair mechanisms in dividing and non-dividing cells. DNA Repair (Amst). 2013;12:620–36.
15. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs RA, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333:1154–7.
16. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333:1157–60.
17. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, Vostrov A, Abdullaev Z, Lobanenkov V, Gray A, Liu C, Chang SS, Ostrow KL, Westra WH, Begum S, Dhara M, Califano J. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PLoS One. 2009;4:e4961.
18. Chang SS, Jiang WW, Smith I, Poeta LM, Begum S, Glazer C, Shan S, Westra W, Sidransky D, Califano JA. MicroRNA alterations in head and neck squamous cell carcinoma. Int J Cancer. 2008;123:2791–7.

Chapter 2
Molecular Features and Treatment Modalities of "Classic" Versus Human Papilloma Virus-Associated Head and Neck Cancer

Giannis Mountzios and Amanda Psyrri

Abstract *Human papilloma virus* (HPV)-associated squamous cell carcinoma of the head and neck (SCCHN) represents a unique disease entity, with distinct epidemiological and molecular characteristics from those of tobacco- and alcohol-related SCCHN, resulting in different biological and clinical tumor behavior. The implication of HPV in the pathogenesis of a subset of SCCHN patients has generated the hypothesis that vaccine-induced immune response against HPV may provide substantial clinical benefit; however, the impact of prophylactic HPV vaccines on the incidence of HPV-associated SCCHN remains to be clarified. Preliminary evidence also shows that therapeutic HPV vaccines are able to induce potent, HPV-specific, immune responses that correlate with tumor regression, and therapeutic HPV vaccines are currently undergoing intense investigation in early clinical trials, especially in the subset of patients with oropharyngeal cancer. The question of de-escalation of treatment in the favorable prognosis subset of patients with HPV-associated disease is also currently being explored in clinical trials in an effort to minimize unnecessary toxicity without compromising therapeutic efficacy.

Keywords Squamous cell cancer of the head and neck · Human papilloma virus · HPV-related vaccine · Oropharyngeal cancer · Dose de-escalation

2.1 Introduction

Squamous cell carcinoma of the head and neck (SCCHN) represents the sixth most common malignancy and accounts for 6 % of all cancer cases. Approximately 650,000 new cases and 350,000 SCCHN-related deaths are reported worldwide annually [14]. A number of "traditional" clinicopathological parameters such as tumor

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site and tumor, node, metastases (TNM) stage have been used for many years to identify distinct prognostic subgroups among SCCHN patients. Recently, advances in molecular biology together with the development of novel genomic and proteomic approaches have enabled the classification of patients into distinct subgroups defined by distinct etiological and molecular characteristics. Moreover, the gradual elucidation of head and neck tumor biology has led to the identification of molecular pathways associated with malignant transformation and progression of tumor cells and others that may be responsible for resistance to chemotherapy and radiotherapy [19]. Beside modern surgery, sophisticated irradiation techniques and novel chemotherapy strategies for SCCHN, molecular targeting approaches are expected to offer enhanced clinical benefit and diminish normal tissue toxicity in the not very distant future.

Regarding etiological factors, tobacco and alcohol have historically accounted for the vast majority of SCCHN cases [14]. More recently, exposure to certain serological types of *human papilloma virus (HPV)* and particularly type 16 HPV (see further Chap. 3) has been implicated in the pathogenesis of a subset of SCCHN, especially those arising from the tonsillar oropharynx [2, 7, 25]. In parallel to the mounting epidemiological evidence, solid experimental data support a causal association between HPV and a subset of SCCHN in terms of molecular pathology. Rampias et al. [33] have shown that HPV-associated SCCHN is characterized by constant expression of the HPV E6 and E7 viral oncogenes, and that repression of viral oncogene expression hampers the growth or survival of these cancer cells. This finding fueled the expectation that HPV-targeted therapeutic approaches such as vaccines eliciting a cytolytic immune response to cells expressing these oncoproteins might provide clinical benefit, even in the advanced disease setting.

Another critical aspect of HPV-related (HPV+) SCCHN carcinogenesis is the often more favorable prognosis of this subset of patients, as compared to SCCHN patients without evidence of HPV infection [23, 47]. These patients are more treatment responsive. There is also evidence for a slower natural history for HPV-associated cancers, whether the manifestation of this is a longer time to recurrence following chemoradiation, or longer survival in the metastatic setting [17, 22]. This observation raises the question whether these patients can be treated with curative intent by using less toxic interventions. Consequently, an important aim of novel approaches for treating HPV-associated cancers will be to obviate the devastating side effects of intensified treatment developed for poor prognostic subsets, by using less toxic treatment without compromising therapeutic efficacy. In the same context, ongoing clinical trials are studying the potential for de-escalation of radiation therapy duration and/or intensity in HPV+ in the setting of different chemoradiotherapy regimens. The role of the epidermal growth factor receptor (EGFR)-targeting monoclonal antibody cetuximab, often used in combination with radiotherapy to improve therapy of SCCHN [4], will also have to be clarified in the subset of patients with HPV-associated disease in prospective clinical trials. This chapter will briefly contrast the main events of head and neck carcinogenesis in HPV-associated SCCHN versus in SCCHN arising from carcinogenic exposures such as tobacco, with an emphasis on the implications of these carcinogenic mechanisms for the treatment and prevention of SCCHN. The reader is referred to later chapters in this volume for in depth discussion of specific named signaling pathways.

2.2 Molecular Progression Model for "Classic" SCCHN

Recent advances in our understanding of the molecular progression of SCCHN have been provided by two whole-exome sequencing (i.e., sequencing exons of all known protein-encoding genes) studies conducted on approximately 100 predominantly HPV-negative SCCHN specimens, independently by two research groups ([1, 38] and reviewed in Chap. 13). While there was a fivefold difference in the average number of mutations reported per tumor, several key findings were similar in the two studies. Each, together with vast previous genomic and functional analyses of SCCHN, underscores the dominant roles of tumor suppressor pathways including p53, Rb/INK4/ARF, and Notch in disease pathogenesis, while only a small number of oncogenes targeted by activating mutations were identified [1, 38]. These cancer genes play major roles in regulation of cellular proliferation, squamous epithelial differentiation, cell survival, and invasion/metastasis, pathways that are pivotal to the pathogenesis of SCCHN and mainly reflect programs involved in normal development within the stratified squamous epithelium. Given the relative dearth of driver oncogenes in SCCHN, targeting these pathways for cancer therapy represents a major challenge for improving outcomes for this disease.

In tobacco-associated SCCHN, loss of heterozygosity at the 9p21–22 gene loci is the most frequent genetic alteration and occurs even in the earliest definable lesions, including dysplasia and carcinoma in situ [19]. Of note, the p16 (CDKN2) gene is located within this chromosomal region and has been implicated as a candidate tumor suppressor gene that becomes inactivated during malignant transformation [46]. Functional inactivation of p16 protein in SCCHN typically occurs as a result of homozygous deletions or methylation of the 5′ CpG promoter region of p16. In untransformed cells, the p16 protein prevents phosphorylation and subsequent activation of the retinoblastoma protein (pRb) and the additional Rb family members p107 and p130 by the cyclin D1-CDK4/CDK6 complex. Phosphorylated Rb isoforms negatively regulate the activity of E2F transcription factors by forming complexes and subsequently repressing transcription of genes involved in cell cycle progression such as cyclin A; this repression is lost in cells lacking p16 [16]. Tobacco use may serve as a potential cause for loss of p16 function, because p16 inactivation occurs in other smoking-related cancers such as lung cancer. Typically, loss of p16 expression is associated with significantly inferior outcome in SCCHN [16, 46].

Approximately 50 % of SCCHN cases are characterized by TP53 mutations [26]. In the rest of the cases, functional inactivation of TP53 may also occur via other mechanisms, including overexpression or gene amplification of MDM2, encoding a protein that mediates proteasomal degradation of TP53, and deletion of CDKN2A, which eliminates p14/ARF, a negative regulator of MDM2. In preclinical models, functional inactivation of TP53 protein via mutation is associated with progression of preinvasive lesions to invasive SCCHN [26]. The finding that TP53 is mutated in precursor lesions and benign-appearing mucosa provides support to a "patch-field" progression model of SCCHN carcinogenesis, in which the index squamous carcinoma (as well as second primary tumors) develops from a genetically altered

field in the mucosa, arising from a putative stem cell—bearing a mutated TP53 gene—which expands to a clonal patch. Interestingly, in several occasions, the TP53 mutations found in the tumor differ from those in adjacent mucosa, implying that multiple patches give rise to distinct clones and suggesting that primary and recurrent tumors from the same patient could in fact progress from unique clones through independent acquisition of additional genetic alterations [26]. In addition to its tumor-promoting role, TP53 inactivation is also associated with aggressive clinical behavior of tumors, independent of the impact of the response to genotoxic therapy. Therefore, truncating and other disruptive mutations of TP53 are significantly associated with inferior outcome—after primary surgery with or without postoperative radiotherapy—compared with either nondisruptive mutations or no mutation at all ([30], and reviewed in Chap. 9).

Cyclin D1 gene amplification is seen in about one-third of tobacco-induced SCCHN and is usually associated with invasive disease. Elevated expression of cyclin D1 may indirectly upregulate cyclin-dependent kinase (CDK) 2 activity by sequestering the CDK2 inhibitors p21 and p27, or alternatively, cyclin D1 may function as a cofactor by binding to DNA repair proteins (i.e., BRCA2) or transcription factors (PARPγ). Interestingly, although both p16 inactivation and cyclin D1 amplification promote cell cycle progression via activation of CDK4 and CDK6, they are not mutually exclusive genetic alterations. Cyclin D1 protein overexpression is associated with significantly worse survival times in SCCHN [5, 12].

Taken together, in tobacco-associated SCCHN, functional inactivation of p16 protein leads to inactivation of the Rb pathway (one of the earliest events in the carcinogenic progression model). Functional inactivation of p53 tumor suppressor protein occurs mainly via p53 gene mutation. Heavy smokers and individuals with a history of heavy alcohol consumption may present with multiple precancerous and cancerous lesions of the upper aerodigestive tract, due to the phenomenon of field cancerization. Due to this phenomenon, individuals with tobacco-induced oropharyngeal squamous cell carcinoma (OSCC) are also prone to develop second primary cancers [2].

Signaling by phosphoinositol-3-kinase (PI3K) is frequently activated in SCCHN (discussed in detail in Chap. 7). Morris et al. [24] have determined that 74 % of SCCHN contain activating genetic alterations, mainly copy number alterations, affecting components of the PI3K pathway. Activating mutations in the “hot spot” codons (9 and 21) of the PIK3CA gene, encoding the catalytic subunit of PI3K, occur in 6–11 % of SCCHNs [31, 32, 37]. Several inhibitors of components of the PI3K pathway are currently being investigated in clinical trials [11].

The EGFR tyrosine kinase (RTK) is commonly overexpressed or activated in SCCHN and represents the most validated molecular therapeutic target to date [24]. Cetuximab is a chimeric IgG1-human antibody directed against the extracellular domain of EGFR, blocking ligand binding to the receptor, and, as reviewed in Chap. 4, enhances the efficacy of chemotherapy or radiation [4, 6, 44]. Clinical development of cetuximab has been hampered by the lack of robust biomarkers, able to reproducibly predict response to cetuximab. Recently, a probable mechanism of cetuximab resistance has been suggested, involving the presence of mutations that result in constitutive activation of EGFR-mediated signaling. Mutation or amplification of the

mesenchymal-epithelial transition (MET/c-Met) RTK gene or MET protein overexpression has been reported in SCCHN. Importantly, MET has been implicated in cetuximab resistance, and is itself being evaluated as a target (see Chap. 5). Moreover, the small molecule crizotinib, which inhibits both the MET and anaplastic lymphoma kinases (ALK), has recently gained Food and Drug Administration (FDA) approval in non-small cell lung cancers harboring ALK translocations.

In humans, three RAS genes encode four highly homologous RAS proteins: H-RAS, N-RAS, K-RAS4A and K-RAS4B (K-RAS4A and K-RAS4B are splice variants of the K-RAS gene). Activating point mutations in genes encoding the Ras subfamily of small guanosine 5′-triphosphate (GTP)-binding proteins contribute to the formation of a variety of human malignancies. The identification of Ras-related resistance mechanisms to EGFR inhibitors has been extremely important in several solid tumors and mainly in colorectal cancer and seems to be relevant in the clinical management of patients with head and neck cancer as well [24]. While K-RAS mutations are rare (approximately 1 %) in SCCHN, HRAS mutations appear more common, particularly in tobacco chewers—whereas the reverse is true for several other malignancies [1, 38].

Finally, the most novel finding of the recent whole-exome sequencing studies of SCCHN is the identification of mutations within the NOTCH1 gene (12–15 % of SCCHN), and within other NOTCH family members (3–5 %) ([1, 38]; discussed at length in Chap. 15). Notch signaling appears to have a tumor suppressor role in SCCHN since most of the identified mutations were inactivating mutations. Notch has a pivotal role in promoting terminal differentiation in stratified epithelium, by activating suprabasal keratins and through indirect effects on the Wnt, Hedgehog, and interferon response pathways [9], some of which are also emerging as relevant to SCCHN (see Chaps. 10 and 11).

2.3 Molecular Progression Model of HPV-Associated SCCHN

The molecular pathways of neoplastic transformation in HPV-associated SCCHN are clearly distinct from those of “classical” tobacco/alcohol-associated disease. HPVs are small, nonenveloped DNA viruses which give rise to a large spectrum of epithelial lesions with low malignant potential such as “warts” or “papillomas” in both males and females. In recent decades, however, a subset of HPVs, namely the “high-risk” HPVs, have been identified and isolated, which are able to induce the formation of precancerous lesions [14]. Large epidemiological studies have shown that HPVs are almost universally present in humans, but only a small fraction of people infected with high-risk HPVs will eventually develop cancer, and this will happen often decades after the original infection.

The molecular progression model of HPV-driven malignant conversion was first elucidated in cervical cancer, the most extensively studied HPV-associated malignancy and the historically first to be etiologically correlated with HPV infection [3, 15]. Mounting preclinical evidence suggests that the integration of DNA from high-risk HPVs into the cellular genome of the host mitigates the expression of the

transcription/replication factor E2F that functions as a transcriptional repressor of the virally encoded oncogenes, E6 and E7 [40]. E6 binds and degrades p53 through an ubiquitin-mediated process, and E7 binds and destabilizes pRb and related proteins. The subsequent functional inactivation of p53 and pRb tumor suppressor pathways induces genomic instability that triggers neoplastic transformation [33, 42]. In addition, E6 protein interferes with DNA repair enzymes while the E7 oncoprotein can induce chromosomal instability by inhibiting centrosome synthesis and causing alterations in structure and number of chromosomes. It is noteworthy, that despite the gradual acquisition of cellular mutations during the process of malignant transformation, induction of repression in E6 and E7 expression in cervical carcinoma cell lines is sufficient and, by any means, is capable to induce cell growth arrest or apoptosis in any stage of this process [34, 42].

Several lines of epidemiologic and preclinical evidence suggest that high-risk HPVs, and especially type 16, are also associated with a subset of OSCC [7]. More specifically, shRNA depletion of E6 and E7 in human the OSCC cell lines 147T and 090 (HPV16 DNA+) and 040T (HPV DNA negative) led to restoration of p53 and pRb protein expression, increased expression of p53-target genes (i.e., p21 and FAS), decreased expression of genes whose expression is increased in the absence of functional pRb (i.e., DEK and B-MYB), and induced substantial apoptosis, specifically in HPV16+ cells [35].

In contrast to the situation in cervical cancer, transcription of HPV16 E6/E7 messenger RNA (mRNA) in tonsillar carcinomas can occur in the absence of HPV DNA integration, and the virus may exist predominately in an episomal form, underscoring thus the distinct molecular mechanisms of the two malignancies [20]. At present, it remains unclear how the virus maintains its capacity to propagate in host cancer tissues in an episomal form with a high copy number. In one interesting study by Van Tine et al. [42], the authors showed that the HPV E2 oncoprotein may be used as an “anchor” to bind episomal HPV to cellular mitotic spindles, creating thus the necessary molecular “bridge” that will enable viral DNA integration and propagation in the cellular host.

As mentioned above, in tobacco-induced OSCC, abrogation of p53 and retinoblastoma pathways occurs mainly via mutation and genetic/epigenetic alterations, respectively, whereas in HPV-associated disease, functional inactivation of p53 and pRb pathways by the viral oncoproteins obviates the need for mutational inactivation of p53 and pRb genes [42]. However, Strati and colleagues used transgenes that provide direct expression of the HPV16 E6 and E7 proteins to the head and neck tissues of mice and reported that a conditional deletion of Rb in the same tissues was not able to reproduce all E7-mediated phenotypes [39]. The authors concluded that pRb-independent effects of E7 may also play a critical role in head and neck carcinogenesis, and further research towards this direction is warranted.

Another molecular pathway that has been implicated in E6 and E7-driven oncogenic activation is the Wnt/β-catenin pathway [9]. Repression of the E6/E7 genes in HPV16+ oropharyngeal and cervical cell lines (SiHa and HeLa) [34] substantially reduced nuclear beta-catenin levels. The protein levels of beta-catenin are tightly regulated by the ubiquitin/proteasome system; the study showed that HPV-dependent

changes in the activity of the E3 ubiquitin ligase seven in absentia homologue (Siah-1) is involved in the nuclear accumulation of beta-catenin and activation of Wnt signaling.

It must be emphasized that HPV DNA detection by itself in a SCCHN sample does not per se prove causal association between the virus and the disease. Only transcriptionally active HPV DNA is biologically and clinically relevant in the development of SCCHN. On this basis, the incidence and clinical implications of biologically relevant HPV16 infection was studied in a cohort of 107 OSCCs treated with primary radiotherapy or surgery followed by postoperative radiotherapy [33]. HPV16 DNA viral load was determined by quantitative polymerase chain reaction (PCR) and, additionally, corresponding tissue arrays were constructed in order to study the expression of p53, pRb, and p16 proteins using a quantitative in situ method of protein analysis (AQUA). The study disclosed 3 separate tumor classes with distinct molecular and clinical features based on HPV16 DNA presence and p16 expression status: one class of HPV16-negative/p16 nonexpressing, one class of HPV16-positive/p16 nonexpressing, and one class of HPV16-positive/p16 expressing oropharyngeal tumors [33]. As expected, 5-year overall survival (OS) in class III was significantly better (79 % compared to 20 and 18 % for classes I and II, respectively, $p - 0.0095$). Disease-free survival for the same class was 75 % versus 15 and 13 % for the other two classes ($p - 0.0025$). Similarly, the 5-year local recurrence was 14 % in class III versus 45 and 74 % ($p - 0.03$). Only patients in class III had significantly lower p53 and pRb expression ($p - 0.017$ and 0.001, respectively). Multivariable survival analysis confirmed the prognostic value of the 3-class model. This pivotal study demonstrated that only the HPV16-positive/p16 expressing tumors "resemble" the cervical carcinogenesis model biologically, and confirmed that they are the ones associated with significantly better prognosis [33].

Downregulation of the Notch signaling pathway may play a procarcinogenic role in HPV-associated SCCHN. Notch activity has been associated with suppression of HPV E6 and E7 oncoprotein expression, which may select for loss of Notch signaling in HPV+ SCCHN [41]. An increased incidence of PIK3CA mutations in HPV-related as compared to non-HPV-related tumors was noted in exome sequencing studies [1, 38]. It has been suggested that PIK3CA mutations may synergize with the HPV E6 and E7 oncoproteins in the development of invasive oropharyngeal cancer, similar to cervical carcinoma [11]. Finally, HRAS mutations often occur in HPV-driven tumors, suggesting that HRAS mutations may also synergize with HPV E6 and E7 in tumor promotion.

2.4 HPV-Targeted Therapy

As is extensively reviewed elsewhere in this volume, emerging data on loss of tumor suppressor function in HPV-negative SCCHN, dissection of treatment resistance pathways, and synthetic lethality studies guide the selection of novel therapeutic targets in these cancers. In contrast, HPV-associated cancers are quite sensitive to existing therapies. However, increasing sophistication in our understanding of viral

oncogenesis also opens up the possibility of therapies specific for virally induced SCCHN, including therapeutic vaccines.

Prophylactic Vaccines HPV-associated SCCHN can theoretically be prevented by vaccines designed to induce appropriate HPV-specific immune responses. The conceptual basis for a prophylactic vaccine is to stimulate the immune system to elicit a neutralizing antibody response prior to or upon exposure to high-risk HPVs so as to prevent establishment of persistent infection. HPV capsid structural proteins displayed on the viral surface represent the most common neutralizing epitopes for antibody production. As inhibition of virus attachment to host cells normally prevents integration of oncogenic viral DNA to the cellular host DNA, targeting these surface proteins should abrogate the development of invasive cancers induced by high-risk HPVs.

In this context, recombinant HPV virus-like particles (VLPs), created by overexpression of major capsid HPV protein L1, possess the capacity to mimic authentic virions but are noninfectious. Consequently, immunization of animals with VLPs protects from experimental infection with the homologous animal papilloma virus [41]. Using this application, two HPV prophylactic vaccines have received approval from the U.S. FDA. The quadrivalent vaccine (Gardasil Merck & Co., Inc.) [28] protects against HPV types 6, 11, 16, and 18. It was first licensed in 2006 for use in females aged 9–26 years old for the prevention of HPV-related cervical [45], vaginal, and vulvar [18] cancers. Additional clinical trial data demonstrated the effectiveness of the vaccine in preventing genital warts in males, and in 2009, clinical indications for the vaccine were expanded to include males in the same age range (Centers for Disease Control and Prevention, CDC). More recent studies have also demonstrated the effectiveness of the vaccine in prevention of anal cancer [29], and thus licensure was further expanded to also include anal cancer prevention. Notably, the vaccine has been particularly efficacious (98 %) for the prevention of anal, cervical, vaginal, and vulvar cancers in HPV16/18-naïve individuals. As expected, vaccine efficacy was lower (50–78 %) when analysis was restricted to individuals previously infected with high-risk HPVs.

The second HPV vaccine, Cervarix® (HPV2), is a bivalent vaccine that protects against HPV types 16 and 18. This vaccine was approved for use in the USA in 2009 for the prevention of cervical cancers (U.S. FDA, 2009). Since it does not cover low-risk (6, 11) HPV types, it is not effective for the protection against genital warts. Similar to the quadrivalent vaccine, the bivalent vaccine is very effective (97 %) in the prevention of HPV16/18-associated cervical cancers in HPV-naïve individuals, but a lower efficacy is observed when women already infected with HPV16 or 18 are included in the analyses. In contrast to the quadrivalent vaccine, the bivalent vaccine has not been tested in clinical trials for efficacy against other HPV-associated cancers, such as those of vagina, vulva, or anus. Nevertheless, structural similarities between the two vaccines indicate that the bivalent one might also be effective in preventing other cancers of the anogenital region, etiologically linked to HPV16 and18.

The fact that the vast majority of HPV-associated OSCCs are caused by HPV16 suggests that both vaccines might be particularly effective in preventing HPV-associated OSCC [20]. However, the impact of these vaccines on the incidence of

persistent oral HPV infection has not been studied in prospective clinical trials. Data from animal models immunized against HPV16 have demonstrated a reduction in the development of HPV oral lesions. Nevertheless, it remains to be clarified whether persistent oral HPV infection can trigger neoplastic transformation and induction of precancerous lesions in oropharyngeal mucosa, as it does in the case of cervical carcinoma [13]. The natural history of oral HPV infection has not been fully elucidated and routine screening for HPV-associated OSCC is not currently recommended. The situation is further complicated by the fact that most HPV-directed vaccine clinical trials use cervical precancerous lesions as the optimal endpoint due to the long interval between HPV infection and development of invasive cervical cancer [36]. The same endpoint is not easily applicable to OSCC, as the stages of precancerous lesion development and evolution have not been as clearly defined.

Despite the optimism heralded by these first two vaccines, several issues should be resolved before wide clinical application of this therapeutic strategy in HPV-related SCCHN : Firstly, the duration of protection is uncertain. Clinical trials of the quadrivalent vaccine that have followed women up to 5 years after vaccination suggest that protection remains high despite the observed decline in neutralizing antibody titers after an initial plateau. Clinical trials of the bivalent vaccine also show durable clinical efficacy reaching up to 6.4 years, but even further follow-up is required. Given the fact that OSCC typically develops during the fifth to sixth decade of life, the impact of the vaccine on the incidence of OSCC still remains to be determined. Longitudinal studies comparing the incidence of OSCC before and after the introduction of the vaccine may answer this question.

Therapeutic HPV Vaccines Several lines of evidence suggest that an intact immune system may be able to entirely eliminate HPV infection from the host. First, most healthy individuals infected with HPV clear the infection without any associated clinical manifestation of symptoms. Only a minority of individuals is not capable of clearing the virus and subsequently develops HPV-associated lesions. Second, immune cell infiltration is often found in HPV-associated regressing lesions while these cell types are absent in persistent disease, providing evidence of enhanced immune reaction in cases of HPV eradication. Last, immunocompromised individuals such as HIV-infected patients have documented higher rates of HPV infection and associated lesions, suggesting that the weakened immune system is unable to eradicate HPV infection from the host [43]. Therapeutic vaccines by definition aim to eliminate HPV-infected cells by developing robust cellular T-cell immune responses that are able to recognize and subsequently eliminate HPV-infected cells.

The HPV16 E6 and E7 proteins represent ideal targets for immunotherapy with curative intent. The fact that they are foreign viral proteins uniquely expressed by the entire virus-infected cell population renders them more immunogenic and target-selective than a self-protein overexpressed in cancer cells. A number of therapeutic modalities have been developed to E6 and E7, including DNA vaccines, viral vector vaccines, bacterial vector vaccines, peptide vaccines, and cell-based vaccines.

DNA vaccines were among the first to be tested in HPV-associated OSCC [20]. A phase I open-label dose-escalation trial of the DNA vaccine pNGVL4a-CRT E7 aiming to elicit immunologic response against HPV16 E7 has been initiated at John

Hopkins University in patients with HPV-associated OSCC (NCT01493154). This promising vaccine uses a targeting strategy that conjugates the nononcogenic derivative of the E7 antigen of HPV16 to the immunostimulatory molecule calreticulin (CRT). In this derivative, E7 is expressed with amino acid substitutions at positions 24 (cysteine to glycine, C24G) and 26 (glutamic acid to glycine, E26G), resulting in omission of the retinoblastoma protein binding site and thus eliminating ability to induce malignant transformation of transfected cells. Strategies to enhance clinical efficacy of vaccines include the use of alternative administration routes and the combination of the vaccine with chemotherapy. For example, administering DNA vaccines via electroporation instead of intramuscular needle injection may increase virus-specific immune responses, as shown in a recent clinical trial of an HIV DNA vaccine [43]. The HPV vaccine described above is administered via electroporation, and low-dose cyclophosphamide is given 1 day before vaccination to reduce overt and potentially hazardous hyperimmune responses [43].

An intriguing point in patients with HPV-related OSCC is the high frequency of T regulatory cells that inhibit cellular immune responses, often found in tumor biopsies. Low-dose administration of the immunomodulator cyclophosphamide may decrease both the population and the intensity of invasion by inhibitory T regulatory cells. As a proof of concept, treating tumor-bearing mice with HPV tumor cells with low-dose cyclophosphamide reduced the frequency of inhibitory T cells, enhancing HPV-specific immune responses against tumors and, importantly, resulting in better long-term survival in tumor-bearing animal models [10]. The alkylating agent cisplatin has also been combined with the HPV DNA vaccine, resulting in smaller tumor diameter and longer survival in tumor-bearing mice [10].

Vaccination with peptides derived from HPV antigenic proteins requires uptake of peptide antigen by dendritic cells and the subsequent presentation of the peptide antigen in association with major histocompatibility complex (MHC) molecules [8]. Most studies on peptide-based vaccines have aimed at enhancing vaccine potency by using adjuncts such as granulocyte macrophage colony stimulating factor (GM-CSF) and montanide ISA-51 [10]. In this context, a therapeutic HPV vaccine consisting of overlapping peptide sequences able to recognize both the E6 and E7 oncoproteins was tested in a phase I trial in patients with vulval intraepithelial neoplasia (VIN) grade III [18]. In all cases, satisfactory vaccine-induced immune responses were elicited and there was a striking 47 % complete response rate 40 weeks after the last vaccination dose.

As mentioned above, HPV+ OSCC cancer is associated with increased expression of p16INK4a [35]. A phase I/IIa study of immunization with a p16INK4a peptide (amino acids: 37–63) combined with the immunoenhancer montanide ISA-51 VG in patients with advanced p16+ HPV-associated cancers (NCT01462838) is ongoing. The study attempts to answer the question of whether the p16INK4a peptide can be safely administered and whether it can induce a p16INK4a-specific T-cell immune response in patients with advanced HPV- and p16INK4a-positive head and neck cancer.

The strategy of peptide immunization using epitopes derived from the processing of viral proteins in a specific MHC context is hampered by the fact that only a fraction of the patient population usually expresses the appropriate MHC allele, inhibiting

thus the corresponding T cell response. Furthermore, the rapid extracellular proteolysis of short peptides limits the clinical efficacy of traditional peptide vaccines. In an effort to overcome these problems, a new generation of peptide-based vaccines named “Trojan peptide vaccines” has been developed. Named after the famous Trojan horse, these agents consist of large peptides that contain a sequence derived from the membrane-penetrating human immunodeficiency virus transactivator of transcription (HIV-TAT) moiety fused to furin-cleavable linkers that join viral peptide epitopes that engage multiple HLA-I and HLA-II MHC proteins [27]. This specific Trojan peptide sequence enables the entire peptide to translocate through the cell membrane to the endoplasmic reticulum and Golgi apparatus where the endopeptidase furin resides. Subsequent protein cleavage generates multiple human leukocyte antigen (HLA)-I- and HLA-II-interacting peptides, originating from the Trojan peptide carrier, which become the active, immunosensitizing molecules. A phase I study of immunization with a therapeutic peptide vaccine using two novel Trojan peptide complexes composed of Melanoma antigen E (MAGE-A3) and HPV16 epitopes is ongoing (NCT00257738). MAGE-A3 is of particular interest because of its established overexpression in SCCHN and the availability of well characterized HLA-I and HLA-II epitopes. In this study, Trojan peptides are resolubilized in Montanide ISA 51 and GM-CSF before injection, in order to promote dentritic cell migration and propagation to the site of vaccination.

2.5 De-escalation Strategies: Determining Therapy Based on HPV Status

In the last decade, it has become evident that patients with HPV-associated OSCC have better prognosis compared to their age- and stage-matched counterparts [2]. More specifically, it has been calculated that HPV positivity confers a 60–80 % reduction in risk of death from cancer compared to similarly treated HPV-negative tumors. The absolute survival difference between HPV-positive and -negative tumors is consistently higher than 30 % across prospective studies. The fact that HPV+ OSCC is more responsive to chemotherapy and radiation as compared to HPV-negative cases has raised the possibility that organ preservation strategies may be more successful in these patients. An important aim of novel approaches for a favorable prognosis of HPV-associated cancers will be to obviate the need for intensified treatment developed for poor prognostic subsets.

Given the fact that irradiation is accompanied by both acute and late, chronic treatment-related toxicities, several de-escalation protocols have been developed in an effort to reduce radiotherapy intensity and subsequent toxicity. The Eastern Cooperative Oncology Group (ECOG) phase II study E1308 evaluated whether very platinum-responsive HPV-associated oropharynx cancers, as determined by clinical response to induction chemotherapy, can safely be treated with a lower dose (54 Gy) of intensity-modulated radiation therapy and concurrent cetuximab. Patients who failed to achieve a complete response received standard dose (69.3 Gy)

intensity-modulated radiation therapy (IMRT) with concurrent cetuximab [21]. One-year progression-free survival exceeds 90 % for patients who received reduced-dose radiation, although in the context of the longer natural history of HPV-associated oropharynx cancers, more mature data are necessary before drawing conclusions about the durability of these responses. In the same context, the Radiation Therapy Oncology Group (RTOG) Study 1016 was planned as a noninferiority phase III study that will evaluate whether cisplatin can be substituted with cetuximab with concurrent irradiation employing accelerated IMRT (70 Gy/6 weeks) and aims to determine if the bio-radiation strategy achieves similar survival with less toxicity. Regarding patients with HPV+ OSCC, the recently launched “DE-ESCALaTE HPV” is a multicenter randomized phase III study that will compare cetuximab and concurrent radiotherapy (bio-radiotherapy strategy) to standard concurrent cisplatin chemo-radiotherapy exclusively in patients with HPV-associated OSCC. Of note, the primary endpoint of the latter study is the incidence of acute and late toxic events. Finally, two other studies (NCT1088802/J0988 and NCT01221753) are evaluating additional de-escalation protocols: The former is a phase I/II study of radiation deintensification with concomitant chemotherapy in the favorable subset of HPV+ OSCC, while the latter investigates the use of induction docetaxel/cisplatin/5-fluorouracil (TPF) chemotherapy followed by concomitant chemoradiotherapy with a modified radiotherapy protocol in HPV-related locally advanced OSCC.

2.6 Conclusions

HPV-associated SCCHN represents a unique disease entity, with distinct epidemiological and molecular characteristics from tobacco- and alcohol-related SCCHN, resulting in different biological and clinical tumor behavior. The impact of prophylactic HPV vaccines on the incidence of HPV-associated SCCHN is promising but remains to be clarified. Clinical trials show that therapeutic HPV vaccines are able to induce potent, HPV-specific, immune responses that correlate with tumor regression. Therapeutic HPV vaccines are currently undergoing intense investigations in early clinical trials, especially in the subset of patients with OSCC. The question of de-escalation of treatment in the favorable prognosis subset of patients with HPV-associated disease is also currently being explored in clinical trials in an effort to minimize unnecessary toxicity without compromising therapeutic efficacy.

References

1. Agrawal N, Frederick MJ, Pickering CR, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–7.
2. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24–35.
3. Bonner JA, Ang K. More on severe cutaneous reaction with radiotherapy and cetuximab. N Engl J Med. 2007;357(18):1872–3.

4. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354(6):567–78.
5. Braakhuis BJ, Snijders PJ, Keune WJ, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. J Natl Cancer Inst. 2004;96(13):998–1006.
6. Burtness B, Goldwasser MA, Flood W, et al. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005;23(34):8646–54.
7. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29(32):4294–301.
8. D’Souza G, Dempsey A. The role of HPV in head and neck cancer and review of the HPV vaccine. Prev Med. 2011;53 Suppl 1:S5–11.
9. Duan L, Yao J, Wu X, Fan M. Growth suppression induced by Notch1 activation involves Wnt-beta-catenin down-regulation in human tongue carcinoma cells. Biol Cell. 2006;98(8):479–90.
10. Emens LA, Asquith JM, Leatherman JM, et al. Timed sequential treatment with cyclophosphamide, doxorubicin, and an allogeneic granulocyte-macrophage colony-stimulating factor-secreting breast tumor vaccine: a chemotherapy dose-ranging factorial study of safety and immune activation. J Clin Oncol. 2009;27(35):5911–8.
11. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer. 2009;9(8):550–62.
12. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008;100(4):261–9.
13. Garland SM, Smith JS. Human papillomavirus vaccines: current status and future prospects. Drugs. 2010;70(9):1079–98.
14. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92(9):709–20.
15. Goodwin EC, Yang E, Lee CJ, et al. Rapid induction of senescence in human cervical carcinoma cells. Proc Natl Acad Sci U S A. 2000;97(20):10978–83.
16. Hayward SD, Liu J, Fujimuro M. Notch and Wnt signaling: mimicry and manipulation by gamma herpesviruses. Sci STKE. 2006;2006(335):re4.
17. Huang SH, Perez-Ordonez B, Weinreb I, et al. Natural course of distant metastases following radiotherapy or chemoradiotherapy in HPV-related oropharyngeal cancer. Oral Oncol. 2013;49(1):79–85
18. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med. 2009;361(19):1838–47.
19. Leemans CR, Braakhuis BJ, Brakenhoff RH, et al. The molecular biology of head and neck cancer. Nat Rev Cancer. 2011;11(1):9–22.
20. Maeda H, Kubo K, Sugita Y, et al. DNA vaccine against hamster oral papillomavirus-associated oral cancer. J Int Med Res. 2005;33(6):647–53.
21. Marur S, Li S, Cmelak A, et al, E 1308: A phase II trial of induction chemotherapy (IC) followed by cetuximab with low dose versus standard dose IMRT in patients with human papilloma virus (HPV)-associated resectable squamous cell carcinoma of the oropharynx (OPSCC). J Clin Oncol. 31, 2013 (suppl; abstr 6005).
22. Mehra R, Egloff AM, Li S, et al. Analysis of HPV and ERCC1 in recurrent or metastatic squamous cell carcinoma of the head and neck (R/M SCCHN). J Clin Oncol. 31, 2013 (suppl; abstr 6006).
23. Mellin H, Dahlgren L, Munck-Wikland E, et al. Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer. Int J Cancer. 2002;102(2):152–8.
24. Morris LG, Taylor BS, Bivona TG, et al. Genomic dissection of the epidermal growth factor receptor (EGFR)/PI3K pathway reveals frequent deletion of the EGFR phosphatase PTPRS in head and neck cancers. Proc Natl Acad Sci U S A. 2011;108(47):19024–9.

25. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus-induced oncogenesis. J Virol. 2004;78(21):11451–60.
26. Nees M, Homann N, Discher H, et al. Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. Cancer Res. 1993;53(18):4189–96.
27. Nickoloff BJ, Qin JZ, Chaturvedi V, et al. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. Cell Death Differ. 2002;9(8):842–55.
28. Olsson SE, Villa LL, Costa RL, et al. Induction of immune memory following administration of a prophylactic quadrivalent human papillomavirus (HPV) types 6/11/16/18 L1 virus-like particle (VLP) vaccine. Vaccine. 2007;25(26):4931–9.
29. Palefsky JM, Giuliano AR, Goldstone S, et al. HPV vaccine against anal HPV infection and anal intraepithelial neoplasia. N Engl J Med. 2011;365(17):1576–85.
30. Poeta ML, Manola J, Goldwasser MA, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357(25):2552–61.
31. Qiu W, Schönleben F, Li X, Ho DJ, Close LG, Manolidis S, Bennett BP, Su GH. PIK3CA mutations in head and neck squamous cell carcinoma. Clin Cancer Res. 2006;12(5):1441–6.
32. Qiu W, Tong GX, Manolidis S, Close LG, Assaad AM, Su GH. Novel mutant-enriched sequencing identified high frequency of PIK3CA mutations in pharyngeal cancer. Int J Cancer. 2008;122(5):1189–94.
33. Rampias T, Sasaki C, Weinberger P, et al. E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells. J Natl Cancer Inst. 2009;101(6):412–23.
34. Rampias T, Boutati E, Pectasides E, et al. Activation of Wnt signaling pathway by human papillomavirus E6 and E7 oncogenes in HPV16-positive oropharyngeal squamous carcinoma cells. Mol Cancer Res. 2010;8(3):433–43.
35. Rischin D, Young RJ, Fisher R, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol. 2010;28(27):4142–8.
36. Romanowski B. Long term protection against cervical infection with the human papillomavirus: review of currently available vaccines. Human vaccines. 2011;7(2):161–9.
37. Rothenberg SM, Ellisen LW. The molecular pathogenesis of head and neck squamous cell carcinoma. J Clin Invest. 2012;122(6):1951–7.
38. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60.
39. Strati K, Lambert PF. Role of Rb-dependent and Rb-independent functions of papillomavirus E7 oncogene in head and neck cancer. Cancer Res. 2007;67(24):11585–93.
40. Tan TM, Ting RC. In vitro and in vivo inhibition of human papillomavirus type 16 E6 and E7 genes. Cancer Res. 1995;55(20):4599–605.
41. Tumban E, Peabody J, Peabody DS, et al. A pan-HPV vaccine based on bacteriophage PP7 VLPs displaying broadly cross-neutralizing epitopes from the HPV minor capsid protein, L2. PloS one. 2011;6(8):e23310.
42. Van Tine BA, Dao, Wu SY, et al. Human papillomavirus (HPV) origin-binding protein associates with mitotic spindles to enable viral DNA partitioning. Proc Natl Acad Sci U S A. 2004;101(12):4030–5.
43. Vasan S, Hurley A, Schlesinger SJ, et al. In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PloS one. 2011;6(5):e19252.
44. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N, Hitt R. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116–27.
45. Villa LL, Costa RL, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. 2005;6(5):271–8.

46. Weinberger PM, Yu Z, Haffty BG, et al. Prognostic significance of p16 protein levels in oropharyngeal squamous cell cancer. Clin Cancer Res. 2004;10(17):5684–91.
47. Weinberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favorable prognosis. J Clin Oncol. 2006;24(5):736–47.

Chapter 3
Epidemiology of HPV in Head and Neck Cancer: Variant Strains, Discrete Protein Function

Camille C. R. Ragin and Jeffrey C. Liu

Abstract Human papillomavirus (HPV) infection has emerged as a major causative agent for cancers of the head and neck. HPV-positive cancers of the oropharynx are associated with better survival rates than HPV-negative cancers of the oropharynx, which appears likely to be associated with differences in the biology of the two diseases. We will discuss the role of HPV-encoded proteins in host infection and carcinogenesis. HPV infection is increasing worldwide, with recent data showing a dramatic increase in HPV-associated oropharyngeal head and neck cancer incidence, and contrasting infection rates in developed and developing nations. Finally, we will review the emerging biology of intratypic variants of HPV, with different variants associated with different potential for malignancy, and suggest potential areas of further study.

Keywords Human papillomavirus · Oropharyngeal cancers · Variants · Incidence · Mortality

3.1 Introduction

3.1.1 Human Papillomavirus (HPV)

Human papillomaviruses (HPVs) are a family of 8 kb, circular DNA viruses with tropism to basal cells of the epithelial mucosa [1]. To date, over 150 discrete genotypic variants have been described among the human papillomaviridae. These fall into broad subcategories that reflect the type of epithelial cells they are able to infect, e.g., cutaneous versus mucosal cells. HPVs are also classified according to their ability to transform epithelial cells. High-risk HPV genotypes such as HPV16, 18,

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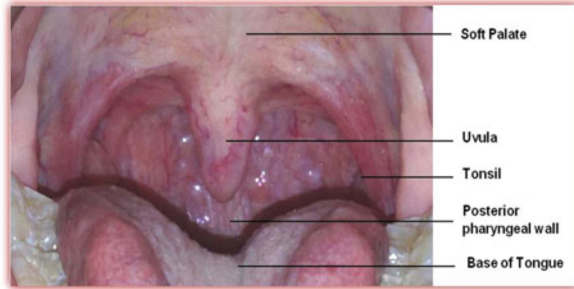

Fig. 3.1 Sub-sites of the oropharynx include the base of tongue (behind the circumvalate papillae of the tongue), soft palate, uvula, tonsil, posterior pharyngeal wall as well as the vallecula (not visible) and lateral pharyngeal wall (not visible)

31, 45, and others are capable of transforming mucosal epithelial cells and inducing malignant lesions, while low-risk HPV genotypes such as HPV6, 11, and others are associated with benign lesions such as warts or condylomas. Benign lesions in the oral cavity are common, and most often involve HPV6 and 11. These HPV genotypes are also often associated with uncommon benign conditions in the larynx such as laryngeal papillomatosis and laryngeal polyps. The involvement of HPV in head and neck carcinogenesis was first proposed in 1983 by Syrjanen et al. [2] based on morphological and immunohistochemical evaluation of oral squamous cell carcinomas, which showed features typical of HPV lesions and were positive for immunoperoxidase staining with anti-HPV serum. This evidence was further corroborated in an epidemiologic study reported by Gillison et al. [3] where HPV genomes were detected in tumors from patients diagnosed with new and recurrent head and neck squamous cell carcinomas. In head and neck cancers of the oral cavity, oropharynx, larynx, and hypopharynx, high-risk HPV infections account for approximately 20–25 % of these lesions. Among these sites, the tumors that arise in the oropharynx carry the largest burden of HPV infections (estimated at 36 %) [4], and HPV16 is the most common genotype isolated [3], [5–24]. Oropharyngeal cancer sites include the tonsil, base of tongue, soft palate, uvula, vallecula as well as the lateral and posterior walls of the oropharynx (Fig. 3.1).

3.2 HPV Proteins and Functions (E1, E2, E4, E5, E6, E7, L1, L2)

The HPV genome encodes seven early (E) genes (E1, E2, E4, E5, E6, E7, E8), and two late (L) genes (L1 and L2). Together with a noncoding upstream regulatory region (URR), these play important roles in viral replication and transcription. Phylogenetic analysis of the open-reading frames of early genes from various HPV genotypes show that proteins from viruses associated with cancer cluster into a single group, suggesting that the genetic basis for oncogenicity is dependent upon the DNA sequences in the early region of the viral genomes [25]. Among high-risk HPV

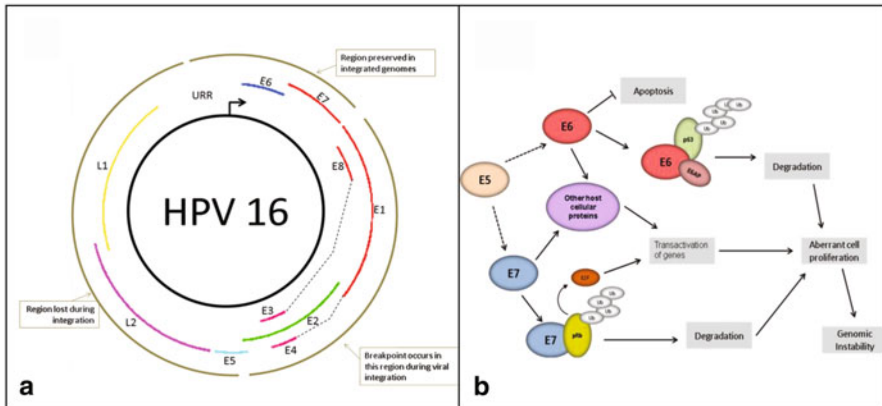

Fig. 3.2a Cartoon of the HPV16 genome (*black*). The upstream regulatory region (URR) contains the enhancer elements for cell transcription factors, and E1 and E2 binding sites. The open reading frames for each early (E) and late (L) gene are depicted as colored lines outside of the circular genome. The dotted lines depict the splice variants E1^E4 and E8^E2 which are derived from fused transcripts from the E1 and E4 or E8 and E2 open reading frames. The *grey lines* depict the regions of the viral genome that are preserved during integration, regions where breakpoints occur during integration and a region that is lost during integration. **b** Interaction of high-risk HPV oncogenes E5, E6 and E7 with multiple host cellular proteins including p53 and pRb. These interactions result in a series of events leading to the inhibition of apoptosis, altered cell proliferation and ultimately genomic instability which leads to cancer development

genotypes, only the early genes E5, E6, and E7 play important roles in viral carcinogenicity (Fig. 3.2a). The differences in oncogenic potential between high-risk and low-risk HPVs are related to differences in the biochemical activities of the early viral gene products.

During the HPV life cycle, the virus invades damaged areas of stratified epithelium and targets the basal cells by binding to a cellular receptor. Heparin sulfate proteoglycan, a linear polysaccharide found in human and animal tissues, has been suggested to be the primary cellular receptor for initial attachment of the HPV virus, and facilitates binding to an unidentified secondary receptor prior to entry [26], [27]. Once internalized, the virus uncoats and the viral DNA is transported to the nucleus. Infections can be nonproductive (i.e., the HPV genome is maintained in episomal form within the cell at low copy numbers) or productive (i.e., viral HPV DNA is replicated and packaged into intact infectious viral particles). Since the HPV genome does not encode for proteins required for viral DNA replication (such as DNA polymerase and other enzymes), the virus must rely on the infected cell's DNA replication machinery to induce viral replication. During the nonproductive phase of infection, the HPV genome is established as an extrachromosome (or episome) and remains tethered to the host chromosome so that it is maintained and segregated into daughter cells after cell division [28].

The switch between nonproductive and productive infections is dependent upon the host cell differentiation state. During productive infection, the transcription of

HPV gene products is tightly regulated by the differentiation-specific gene expression profile of the infected cell. Upon activation of HPV DNA replication, amplification, and packaging of intact viral particles [29], infectious virions egress from terminally differentiated epithelial cells that comprise the cornified (outer surface) epithelium. HPV-associated carcinogenicity is not a normal event that occurs as part of the productive viral life cycle, as this typically involves the extrachromosomal viral genome. Rather, during carcinogenesis, the circular viral genome linearizes and integrates itself into the host chromosome, losing coding sequences for key regulatory genes during the linearization process. A more detailed description of the roles of specific HPV-encoded proteins in the HPV life cycle, including chromosomal integration and viral carcinogenicity, is provided below.

3.2.1 HPV L1 and L2

The L1 and L2 proteins encode the structural components of the virus and are only transcribed in productively infected cells. These structural proteins assemble to form the viral capsid, which comprises 72 "capsomeres". Each capsomere includes a pentamer of the L1 protein, and an L2 protein monomer, which docks into the center of each L1 pentamer. The L1 capsid protein is capable of self-assembling into virus-like particles (VLPs), even in vitro. While essential structural elements of the sequence of the L1 protein are well conserved, a number of surface loops are distinct for different HPV genotypes. Exploiting these features, researchers have used HPV L1 VLPs to generate genotype-specific vaccines including a recombinant quadrivalent vaccine, targeting genotypes 6, 11, 16, and 18 (Gardasil, Merck & Co. Inc.), and another recombinant HPV vaccine targeting genotypes 16 and 18 (Cervarix, Glaxo Smith Kline, provided as an adjuvanted, adsorbed form).

As the virion binds to its receptor, a conformational change occurs that exposes the N-terminus of the L2 protein, allowing it to be cleaved by the protease furin. This leads to the exposure of additional regions of the L2 protein, which subsequently binds to an unidentified secondary receptor, followed by internalization of the viral particle via clathrin or caveolin-mediated endocytosis. For establishment of the infection, binding of the L2 protein to the HPV viral DNA enables its transport into the nucleus. The expression of the L2 protein later in the course of HPV infection is also important for the packaging of progeny viral genomes during viral particle assembly.

3.2.2 HPV E1, E2, E4, and E8

The early proteins E1 and E2 are among the first viral proteins expressed in an infected cell. Both are DNA-binding proteins that regulate viral replication and gene expression. E1 is a DNA helicase/ATPase that is responsible for bidirectional unwinding of viral DNA to facilitate viral genome replication. The E2 protein is a transcriptional regulator that binds at viral transcription factor binding sites within

the URR to activate or repress transcription of HPV genes, with the viral oncogenes E6 and E7 particularly dependent on E2 activity. The URR is upstream of the early promoter, and contains enhancer elements that are responsive to the binding of host transcription factors such as AP1, Oct-1, SP1, and YY1, and are adjacent to binding sites for the viral transcription factors E1 and E2. The activation and repression of the early promoter is tightly regulated by E2 binding. A low level of E2 binding activates the early promoter, while a high level of E2 blocks the binding of host transcription factors and therefore represses the early promoter. E2 is also responsible for maintaining the viral genome as an extrachromosomal replicon by recruiting the E1 protein to the replication origins and tethering HPV genomes to the host chromosome. The HPV E1 protein has been shown to bind to a number of host proteins such as cyclin-CdK2 [30], Hsp40/Hsp70 [31], SW1/SNF5 [32], histone H1 [33] and Ubc9 [34], but the significance of these interactions is not fully understood.

Heat shock protein (Hsp40) stimulates viral replication by facilitating the formation of E1:hsp40 dihexameric complexes which associate with the viral replication origin and remain associated with the replication elongation complex [31], [35]. The E2 gene also produces an alternatively spliced transcript that encodes E8 fused to a partial sequence of E2 (E8^E2). This splice variant is thought to negatively regulate HPV replication [36], [37]. Functional studies show that the E8^E2 proteins of high-risk HPV16, 18, and 31 inhibit the promoter that drives the expression of the HPV E6 and E7 oncoproteins. The E8^E2 fusion protein also exhibits long-distance transcriptional-repression activities (i.e., represses viral transcription by binding to E2-binding sites distal to the early promoter [37]), and has been shown to inhibit the growth of the cervical cancer cell line HeLa. Consistent with the role of E8^E2 in repression of the viral oncogenes E6 and E7, the expression of E8^E2 results in a rapid increase in the cellular p53 and p21 genes, which negatively regulate cell growth [38]. Among the other early proteins, E4 (expressed as an E1^E4 fusion protein) is known to interact with cytokeratin filaments [39], [40], but its function is currently unknown.

3.2.3 HPV Oncogenes E5, E6, and E7

Three oncogenes are encoded by the early open reading frames of high-risk HPV genotypes (E5, E6, and E7) (Fig. 3.2b). In general, HPV oncoproteins E5, E6, and E7 promote carcinogenicity by interfering with the regulation of cell growth by host cellular proteins, thus inducing genomic instability. The HPV16 E5 protein is the most commonly studied genotype of oncogenic E5. This protein is 90 amino acids in length and has been shown to localize in intracellular membranes such as the Golgi apparatus, endoplasmic reticulum, and nuclear membrane [41], [42]. While the biochemical mechanisms related to HPV16 E5 carcinogenesis have not been clearly elucidated, the oncoprotein is thought to promote carcinogenesis during early stages of the established viral infection, since the gene is often deleted when the HPV genome becomes integrated in the host chromosome [43], [44]. Among its

activities, HPV16 E5 appears to amplify signal transduction pathways by cooperating with HPV16 E7 [45] and activating the epidermal growth factor receptor (EGFR) to stimulate viral gene expression and cell proliferation. The protein also interferes with the recycling of major histocompatibility complex (MHC) Class I and II molecules as well as other receptors to the cell surface, and reduces gap-junction-mediated intercellular communication via dephosphorylation of connexin 43 [46–48]. The inhibition of MHC Class I and II expression is a common immune evasion tactic used by many viruses, while the limitation of gap-junction-mediated intercellular communication results in a deficiency in tissue homeostatic feedback, promoting carcinogenesis.

The E6 protein interacts with a number of host proteins responsible for cell proliferation, with the interaction inducing the degradation of cellular partners. The degradation is induced because E6 also interacts with E6-AP, a ubiquitin ligase, which targets interactive partners for destruction. Many proteins degraded by E6-AP contain PDZ domains; a particularly critical target in head and neck cancer is the tumor suppressor protein p53 [49–51]. In addition, other important interactions of high-risk HPV E6 proteins involve degradation of *NFX1-91*, a transcriptional repressor that regulates telomerase expression, contributing to cell immortalization [52], abrogation of host interferon response by interacting with IFN regulatory factor 3 (IRF3) [53], and interaction with the focal adhesion protein paxillin to disrupt the actin cytoskeleton (a characteristic of transformed cells) [54]. Consequently, these interactions lead to inhibition of p53-mediated apoptosis, inefficient G1/S checkpoint, deregulation of the cell cycle in cells that contain DNA damage and eventually chromosomal instability.

The high-risk HPV E7 protein also induces cellular proliferation based on interactions with a large number of host cellular proteins. Most importantly, its interaction with the tumor suppressor protein pRb results in hyperphosphorylation and ubiquitin-mediated degradation [55] of pRb. This leads to the release of E2F from pRb/E2F complexes, thus promoting transactivation of S-phase-related genes. The inactivation of pRb leads to upregulation of p16/*CDKN2A,* a noted surrogate marker for HPV carcinogenesis [56]. E7 cellular targets also include other growth inhibitory pocket proteins related to pRb (p107 and p130), cyclin E and A, *JUN* the transcription factor, cyclin-dependent kinase inhibitors p21 and p27, as well as the TATA box-binding proteins [57–61]. Cumulatively, these activities, in combination with those of other HPV oncoproteins, lead to genomic instability including chromosome segregation defects such as structural and numerical chromosomal abnormalities [43], [44], cell transformation and carcinogenesis.

3.2.4 HPV Carcinogenesis

The molecular model of HPV carcinogenicity in head and neck cancer has been developed from earlier cervical cancer studies demonstrating that persistence of high-risk HPV infection increases the likelihood of viral integration into the host

chromosome [62–64]. While HPV integration is a random event throughout the human genome, it is thought to occur as a late event with a predilection for DNA fragile sites (regions of the genome that are late replicating and with loose chromatin structures) [65], [66]. These regions are hot spots for DNA breaks. Furthermore, the linearization of the circular HPV genome prior to integration usually occurs with a disruption in the viral E2 sequence. Therefore, integration is not a normal event during the viral life cycle. The loss of E2 expression lifts the repression of the E6 and E7 oncogene promoter. Therefore, in cervical cancer, linearization and integration of HPV results in overexpression of the viral oncogenes HPV E6 and E7. For head and neck cancer, the relationship between physical state and integration of HPV may not be as simple, since variations in HPV integration status have been reported from different studies. HPV-positive oropharyngeal cancers have been found to carry HPV genomes primarily in episomal form, yet viral oncogenes are still expressed [67], while evidence of integrated or episomal forms only or a combination of both have also been reported in the presence of viral oncogene expression [68–72]. This is currently an area of active study.

3.3 HPV Infections in Normal Oral Mucosa

HPV infection is not a rare event, with many infections not leading to cancer either because individuals are infected with nononcogenic strains, or because an effective immune response prevents establishment of a chronic infection. The 2003–2004 National Health and Nutrition Examination Survey (NHANES) evaluated the serum prevalence of antibodies to HPV6, 11, 16, and 18 among 4,303 persons living in the USA, aged 14–59 years. The study was conducted prior to the introduction of HPV vaccination and represents an estimate of natural HPV exposure in the USA. The overall seroprevalence of any of the four HPV genotypes was 22.4 %, with significant differences between males (12.2 %) and females (32.5 %) [73]. The seroprevalence of HPV16 or HPV18 infection among females was also higher than males (females: HPV16—15.6 %, HPV18—6.5 %; males: HPV16—5.1 %, HPV18—1.5 %) and much smaller percentages of persons had serum antibodies for HPV6 and 11 (females: 3.3 %; males: 1.0 %) and both HPV16 and 18 (females: 2.4 %; males: 0.3 %) [73].

A separate NHANES study conducted from 2009 to 2010 reported a higher prevalence of oral HPV infections among men compared to women, examining virus in oral rinse samples collected from 5,501 individuals aged 14–59 years. The overall oral HPV infection rate for any HPV was 6.9 % and the prevalence of any high-risk infections was 3.7 % which was similar to the prevalence of any low-risk infections (3.1 %). The most prevalent HPV type detected was HPV16 (1.0 %). The prevalence of overall oral HPV infection was higher among males (10.1 %) compared to 3.6 % among females; similarly, males also had higher prevalence of oral HPV16 infection (1.6 %) compared to females (0.3 %) [74]. In this study, the potential risk factors for oral HPV infection were sexual behavior (i.e., higher number of lifetime sexual partners)

and current smoking. Oral HPV infection was also more commonly detected among persons who were sexually experienced but did not report practicing oral sex, suggesting that transmission was likely to be related to sexual contact other than oral sex [74]. Overall, both NHANES studies show that there is a contrasting difference in seroprevalence and oral HPV infection between males and females. Females tended to have higher seroprevalence rates from HPV infection and had lower prevalence of oral HPV infection, with the opposite pattern observed in males. It has been postulated that the higher HPV seroprevalence in females might be related to exposures to genital HPV infections [73] which might result in a greater protection from subsequent oral HPV infections [75].

With respect to racial differences, the previously mentioned NHANES study conducted in 2003–2004 revealed that US non-Hispanic blacks appear to have higher overall HPV seroprevalence rates compared to non-Hispanic whites and Hispanics (46.8, 31.9, and 22.6 %, respectively) [73]. However, among males HPV16 seroprevalence appears to be similar between non-Hispanic blacks and non-Hispanic whites (7.0 vs. 5.6 %) while lower rates were reported among Hispanics (1.5 %) [73]. The prevalence of oral HPV infection according to race is currently unknown. Together, these racial and gender disparities may underlie the differing frequencies in HPV-positive versus HPV-negative head and neck cancers in different populations.

3.4 Global Trends in Head and Neck Cancer Incidence and Mortality

Globally, the incidence of head and neck cancer varies by geography and gender. At least part of this variance is likely attributed to differences in the prevalence of tobacco and alcohol use in different populations. Overall, the age-adjusted incidence of head and neck cancer worldwide is 8.1 per 100,000. By geographic region, the highest rate is observed in Europe (ASW Incidence: 10.8 per 100,000) while Middle Africa has the lowest (ASW Incidence: 3.4 per 100,000) [76]. Oceania, the Caribbean Islands, North and South America, as well as Australia and New Zealand have reported estimates that are higher than the worldwide rates, while the rates throughout Africa and Asia are lower than the world wide estimates (Fig. 3.3). Diagnoses world wide are approximately threefold higher in males (12.7 per 100,000) compared to females (3.9 per 100,000) and similar trends are reported for the USA and other countries around the world (Fig. 3.3) [76], [77]. While males continue to bear the burden of disease, for both males and females, the predicted number of new head and neck cancer cases is higher in less developed countries compared to more developed countries irrespective of age at diagnosis (Fig. 3.4). For younger persons ($<$ 65 years), it is predicted that through 2030, the number of new head and neck cancer diagnoses will increase approximately 2-fold for males and 2-fold for females in the developing world yet remain constant for males and females in developed countries. In contrast, for older persons ($\geq$ 65 years), similar increasing trends of new cancer diagnoses are predicted for males and females in developing countries and only for males in

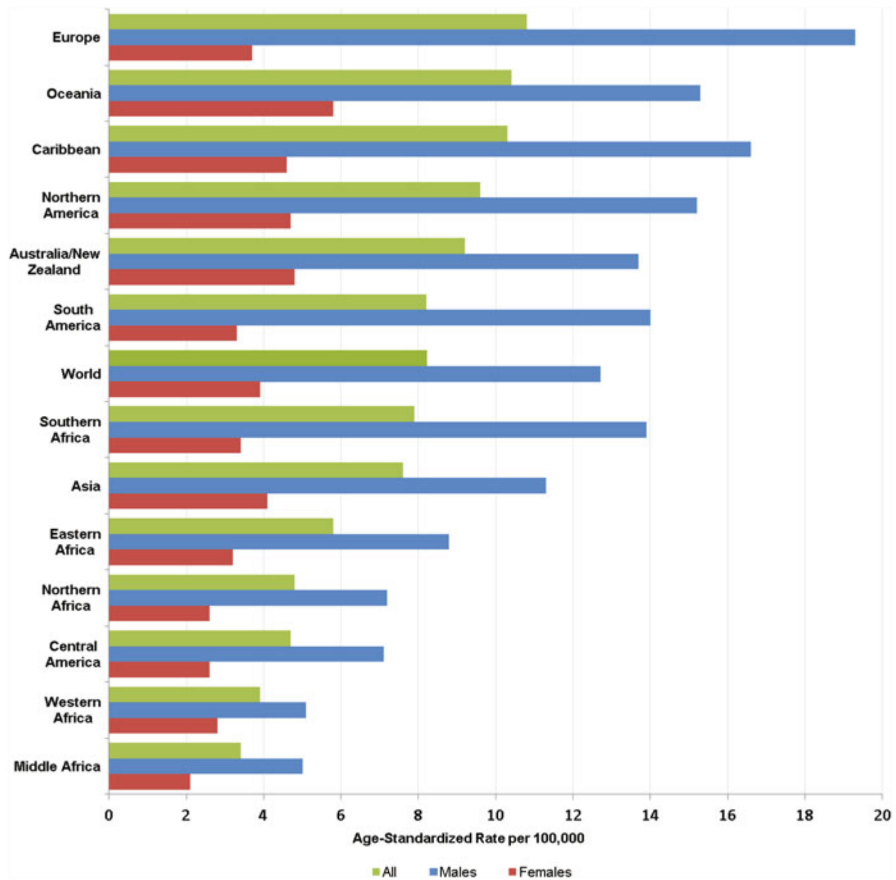

Fig. 3.3 Incidence of head and neck cancer varies by geographic regions and gender, with the highest overall incidence observed in Europe and lowest incidence observed in Middle Africa. Incidence rates in Males (*Blue*) ranges between two to five-fold higher than the incidence rates in Females (*Red*). Graph reflects Age Standardized Rates (World) per 100,000 (Globocan, 2008)

developed countries. These trends may be explained by a number of factors including increasing tobacco use and/or poor oral health in developing countries compared to developed countries [78].

The mortality rate from head and neck cancer worldwide is 4.4 per 100,000. Notably, it is almost fourfold lower for females compared to males (2.0 per 100,000 vs. 7.1 per 100,000) [76] and the reason for this disparity is still unclear. A recent matched analysis reported that 286 female and 286 male head and neck cancer patients with similar clinical and demographic characteristics, diagnosed and treated from a single institution, exhibited no survival disparity [79]. While this study should not be generalized to the general population, it has been speculated that the gender differences in head and neck cancer mortality might be associated with differences

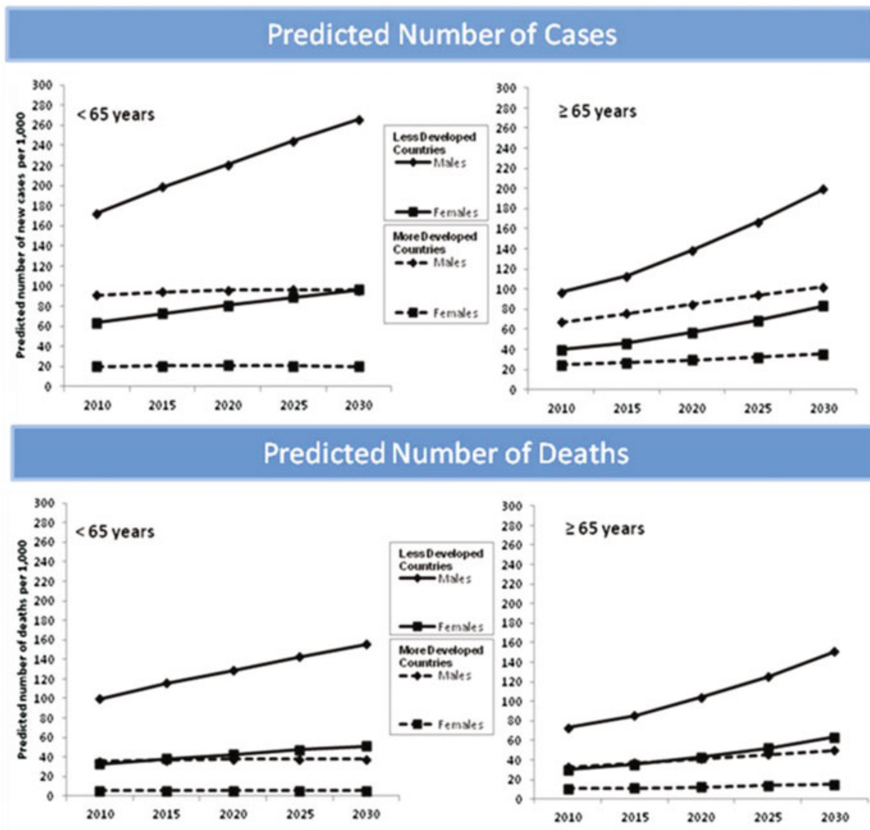

Fig. 3.4 Predicted number of head and neck cancer cases diagnosed and predicted number of deaths: 2010–2030 Globocan (IARC) 2008 are presented as stratified graphs according to age at diagnosis < 65 years and ≥ 65 years. Frequencies for less developed countries (*solid line*) and for more developed countries (*dotted line*) are also presented separately for Males (*diamond*) and Females (*square*)

in incidence based on higher tobacco and alcohol exposure in males compared to females. This still needs further investigation. Overall death rates are highest in the Caribbean Islands. Asia, Europe and Southern Africa also have rates that are higher than the worldwide rate (Fig. 3.5). In contrast, North, South and Central America, Oceania, Australia, New Zealand, and other regions of Africa have death rates lower than worldwide rates. Mortality from head and neck cancer appears to be declining in Europe as well as the US [77], [80] and may be attributed to improvements in treatment and management of head and neck cancer as well as the rising incidence of HPV-related cancers that are more responsive to therapy [81]. Males in developing countries are predicted to carry increasingly the highest burden of deaths from head and neck cancer compared to persons in developed nations, while a slight increase in deaths from this disease is also projected for females in developing countries (Fig. 3.4).

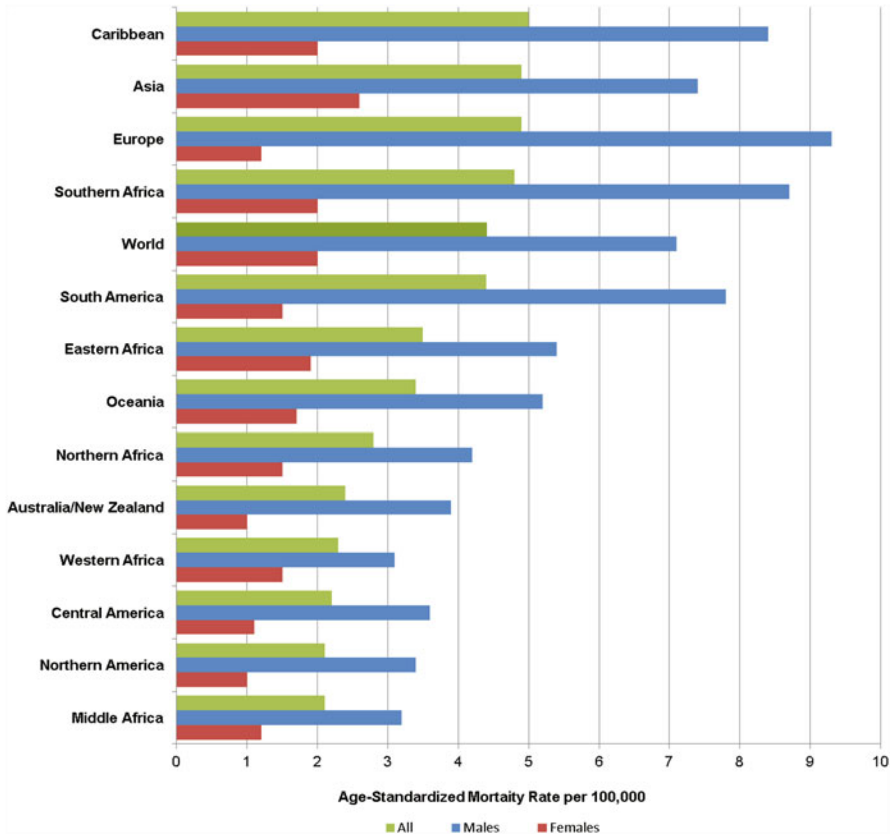

Fig. 3.5 Mortality rates of head and neck cancer according to geographic regions: Age Standardized Rates (World) per 100,000. Mortality rates for Males and Females combined (*green*) are highest in the Caribbean and lowest in Middle Africa. Mortality rates for Males (*blue*) are two to eight times greater than the mortality rates for Females (*red*)

3.5 HPV-positive Head and Neck Cancers

HPV-positive head and neck cancers arise primarily in oropharyngeal sites. The biologic plausibility of HPV carcinogenicity in epithelial cells is well established. There is strength and consistency of association of HPV DNA present in oropharyngeal cancers [82] as well as evidence that the HPV infection is specific to the oropharyngeal cancer cells [3]. This strong epidemiological evidence supports the causal association of HPV with cancers in the oropharynx, and also supports the idea that patients with HPV-positive oropharyngeal cancers have a survival advantage. While there is also evidence of association with viral infection in nonoropharyngeal subsites such as the oral cavity and larynx, there is insufficient evidence to suggest a causal relationship and there does not appear to be a survival advantage for these patients.

3.5.1 HPV-positive Oropharyngeal Cancer

While the majority of head and neck cancers are caused by tobacco and alcohol use, more recently, over the past 10 years, HPV has been demonstrated to be the primary cause of the majority of oropharyngeal cancers while the remaining proportion of oropharyngeal tumors are still attributed to habitual tobacco and/or alcohol use [83]. The virus appears to have an affinity to the epithelial cells of the Waldeyer's tonsillar ring, and therefore is more commonly detected in tumors that arise in the tonsils and other oropharyngeal subsites (Fig. 3.1). It is unclear whether persistent infection is more common in these tissues. Only a portion of HPV DNA-positive oropharyngeal cancers have been shown to be transcriptionally active. Since HPV carcinogenicity relies on the overexpression of HPV oncogenes E6 and E7, it is likely that these tumors might not be attributed to HPV [84], [85]. In developed countries, an increasing trend of oropharyngeal cancers has been observed for several decades, particularly in younger patients [86–89]. It has been suggested that HPV is the driver of this increased incidence since the virus, primarily HPV16, has consistently been detected in these tumors. In Europe, the prevalence of HPV in base of tongue cancers increased from 58 % during 1998–2001 to 81 % during 2004–2007 [90] and similar increases in HPV-positive tonsillar cancers have also been reported (from 23 %, 1970–1979 to 93 %, 2006–2007) [81]. In the USA, for over three decades the prevalence of HPV-positive oropharyngeal cancers has also reportedly increased from 33 % in the 1980s to 63–92 % in the 2000s [91–95].

A meta-analysis published in 2012 confirms this increasing trend of HPV-positive oropharyngeal cancers in all regions of the world, except for Asia, Africa, the Caribbean and South America, where no recent data are available [96]. Various HPV detection methods have been used among these studies, from low-sensitivity assays such as Southern blotting or immunohistochemistry for HPV antigens, to more sensitive methods such as in situ hybridization (ISH) or polymerase chain reaction (PCR). After adjusting for the time period during which the studies were conducted, the increasing trend did not appear to be attributed to improved sensitivity or performance of the HPV detection methods. In addition, the systematic review of published articles shows that prior to 2000, North American studies reported the highest prevalence of HPV-positive oropharyngeal cancers (North America: 50.5 %, Europe: 35.3 %, other countries: 32.2 %). Despite the increasing trend for all geographic regions, after 2005, the pooled prevalence of HPV-positive oropharyngeal cancers was comparable in North American studies (69.7 %) compared to European studies (73.1 %) [96]. This suggests that the prevalence of HPV-positive oropharyngeal cancers diagnosed in Europe appears to be increasing at a faster rate compared to the USA [96]. It is unclear whether this may be due to competing trends such as a greater change in tobacco-related oropharyngeal cancers in the USA vs. Europe. The increase in incidence of HPV-positive oropharyngeal cancers has been observed primarily among white males, in contrast to white women and other race groups where rates have remained unchanged or are decreasing [97–99]. The reason for the greater predilection for HPV-related oropharyngeal cancer in white males is still unclear.

While numerous studies have been conducted on the prevalence of oral HPV infection in cancer-free individuals, it is important to note that the association of oral HPV infection with disease is different from that of HPV in the oropharynx; therefore, one should be careful about correlating the prevalence of HPV infection in the oral cavity mucosa with prevalence of HPV infection in the oropharyngeal mucosa. The presence of HPV in normal oropharyngeal tissue has been reported in a few studies and reports have been variable. In normal and benign oropharyngeal tissues the prevalence of HPV ranges from 0–14 % [15], [100–113]. One study conducted in Belgium evaluated 80 tumor-free tonsils from cancer-free adults and children who underwent tonsillectomy due to sleep apnea or recurrent tonsillitis. The study reported that 12.5 % were positive for high-risk HPV genotypes [111]. Similarly, another study reported that 14 % of 50 cancer-free women from Brazil were positive for high-risk HPV in normal oropharyngeal tissues [112]. A study conducted in the USA evaluated 226 archived palatine tonsils with benign histologic findings that were surgically removed between 1975 and 2001 from adults (> 21 years). This study failed to detect HPV infections in these tissues [113]. In patients with oropharyngeal dysplasia, the prevalence of HPV was reported in one study at 9.4 % [114].

Currently there is insufficient information on the prevalence of HPV in precancerous tissues in the oropharynx and virtually no information on the prevalence rates by gender or race. Some insight has been provided by a few studies that have compared genital HPV infection with HPV status in the oropharynx and suggested the association with sexual behavior. The link between female genital HPV infection and HPV-positive oropharyngeal cancer has been demonstrated, and both male and female cancer patients appear to have increased susceptibility [115–117]. A study of 100 women with abnormal cervical cytology reported high prevalence of high-risk HPV in the oropharynx [118]. This is not surprising since analysis of the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) data (1973–2002) shows that women with cervical cancer diagnoses have an increased risk of developing second primary tumors in the tonsil (Standardized Incidence Ratio (SIR): 3.1) and this risk was in excess compared to women diagnosed with cancers other than cervical cancer [117].

A similar study of SEER data from 1973 to 1994 involving males and females diagnosed with a HPV-associated anogenital cancer (i.e., cervical, vulvar, and anal cancers), also reported an increased risk of second primary cancers in the tonsils for female cervical cancer patients (Relative Risk (RR): 65.2, 95 % confidence interval (CI) = 2.4–10.0) as well as male anal cancer patients (RR: 6.1, 95 % CI = 1.2–17.9) [116]. Furthermore, husbands of invasive or in situ cervical cancer patients also have an excess risk of developing tonsillar cancers (SIR: 2.4 if wife had in situ cervical cancer; SIR: 2.7 if wife had invasive cervical cancer) [119]. A bidirectional association between oropharyngeal cancer and anogenital cancer has also been reported and supports the association of sexual behavior with the development of HPV-associated cancers [115]. The study shows that the risk of developing tonsillar cancer subsequent to anogenital cancer among men who were never married (SIR: 13.0) was much higher compared to men who were married (SIR: 3.8). The acquisition of

genital HPV infection is associated with behaviors such as early age of sexual intercourse and multiple sexual partners; and epidemiological studies have suggested that these behaviors are associated with the transmission of HPV to the oral mucosa [120–122], as well. In addition, the association of these behaviors with the development of HPV-positive oropharyngeal cancers has also been demonstrated [123], [124]. Taking these findings altogether, it is likely that the incidence of HPV-positive oropharyngeal cancer might be related to increased intra- and/or interindividual exposures to genital high-risk HPV infections (sexual and nonsexual); nevertheless, the rising incidence of HPV-positive oropharyngeal cancer among white men still remains to be explained epidemiologically.

Patients with HPV-positive oropharyngeal cancers have improved overall and disease-free survival compared to patients diagnosed with HPV-negative oropharyngeal cancers as well as cancers in nonoropharyngeal sites [94], [125–132]. HPV-positive oropharyngeal cancers have a distinct genetic profile compared to tobacco-related head and neck cancers. The tumors are less likely to carry mutations in *TP53* [84], [85], [133], have differing combinations of allelic losses and gains/amplification [133], [134] as well as a unique host cell transcription profile which involves downregulated expression of interferon-induced proteins such as *IFIT1*, *IFITM1-3*, *IFI6-16*, *IFI44 L*, and *OAS2* and upregulated expression of transcription factors such as *RPA2*, *TAF7 L*, *RFC4*, and *TFDP2* as well as cell division/cycle regulators such as p18, *CDC7*, and p16 [135], [136]. The distinct genetic features of HPV-related and tobacco/alcohol-related cancers are likely associated with the observed difference in patient response to therapy. It is possible that the genetic alterations associated with tobacco-associated carcinogens weigh in more heavily and negatively on the patient's response to therapy. The Radiation Therapy Oncology Group (RTOG) clinical trial 0129 reported that for 266 patients diagnosed with advanced stage oropharyngeal cancer, treated with radiotherapy combined with platinum therapy, the risk of death (categorized into high, intermediate, and low) was dependent primarily upon the HPV status of the tumor followed by the number of pack-years of tobacco smoking, then tumor stage, and nodal stage [137]. The study used an exploratory method, recursive partitioning analysis, which enabled the identification of influential factors related to overall survival. HPV status was the primary determinant for survival in these patients. Patients who were nonsmokers, with HPV-positive tumors and with low nodal stage (N0-N2a) were considered low-risk, while high-risk patients were smokers diagnosed with HPV-negative tumors at tumor stage T4. The intermediate-risk patients were either HPV-positive smokers with high nodal stage (N2b-N3) or HPV-negative nonsmokers with low tumor stage (T2-T3). The 3-year overall survival for low-risk patients was 93 %, for intermediate-risk patients 70.8 %, and for high-risk patients 46.2 % [137]. A recent clinical trial conducted in an independent cohort reported similar results for patients treated with induction chemotherapy followed by chemoradiation or radiation only. In this study, the 3-year overall survival for low-risk patients was 100 %, for intermediate-risk patients 79.6 %, and for high-risk patients 70 % [138]. These findings suggest that this type of risk stratification may help to define clinical decision for appropriate treatment.

A racial disparity in survival has also been noted in head and neck cancer, and particularly among patients diagnosed with oropharyngeal cancer. Black patients have significantly worse survival rates compared to whites. Despite the numerous studies of the impact of HPV on oropharyngeal cancer survival, investigations according to race have been limited. Nevertheless, from the few studies that have been conducted, findings suggest that blacks appear to have a lower prevalence of HPV-positive oropharyngeal cancers. In a multi-center phase III clinical trial of induction chemotherapy followed by concurrent chemoradiation, the overall and disease-free survival of patients diagnosed with stage III/IV head and neck cancer was improved as expected for patients diagnosed with HPV-positive tumors. The subset analysis of 124 oropharyngeal cancer patients (54 black, 70 white) also documented poor median overall survival for blacks compared to whites (25.2 vs. 69.4 months, $p = 0.0006$). There were no noted differences in outcome between blacks and whites diagnosed with nonoropharyngeal cancers. Further analysis of 224 head and neck tumors from 196 white and 28 black patients revealed a significant difference in the prevalence of HPV according to race. Black patients had a significantly lower prevalence of HPV-positive cancers compared to whites (4 vs. 34 %). This study suggests that the racial disparity in oropharyngeal cancer may be attributed to differences in the prevalence of HPV-positive oropharyngeal tumors according to race [139].

The findings from this study have since been complemented by two additional studies. Chernock et al. reported that the poorer disease-free survival among African Americans may be attributed to lower HPV prevalence, the types of treatment and higher tumor stage at diagnoses [140]. Another report by Worsham et al. [141], [142] compared the survival of 118 patients diagnosed with oropharyngeal cancer and found that 51/118 43.2 % were HPV-positive and 67/118 56.8 % were HPV-negative. A lower prevalence of HPV was observed for African Americans compared to white Americans (29 vs. 71 %, $p = 0.024$). The HPV-negative African American patients had poorer survival compared to the HPV-positive African American patients ($p = 0.0012$) as well as the HPV-positive white Americans ($p = 0.0496$). In addition, the HPV-negative African Americans also had poorer survival than the HPV-negative white Americans ($p = 0.0496$) in that study and there was no survival difference between HPV-positive African Americans and HPV-positive white Americans. These findings not only support the important role that HPV plays in oropharyngeal cancer outcome but also suggest that additional factors might be contributing to the poor survival of blacks; therefore, further epidemiological studies addressing racial disparities in oropharyngeal cancer are still needed.

3.5.2 HPV-Positive Nonoropharyngeal Cancer

The role of HPV has also been extensively investigated in studies of head and neck cancers in nonoropharyngeal subsites. The pooled prevalence of HPV-positive nonoropharyngeal cancers for all regions of the world is reported to be approximately 21.8 %, and is higher in Europe (23.7 %) than in North America (12.8 %)

[96]. In contrast to the increasing prevalence of HPV-positive oropharyngeal cancers, HPV-positive nonoropharyngeal cancers do not appear to be increasing, but rather decreasing over time. For studies published prior to 2000, the prevalence of HPV-positive nonoropharyngeal cancers was 22.2 % and decreased to 17.2 % (2000–2004), and 6.2 % (after 2004) [96]. The sensitivity of the detection methods used as well as the type of tissue evaluated did not appear to affect the decreasing trend in HPV prevalence. The reason for this declining prevalence is still unclear.

Nevertheless, studies involving nonoropharyngeal subsites in the oral cavity do suggest a potential association with HPV. In oral cavity dysplasia, 12 studies conducted between 1985 and 2010 show that HPV16 and/or 18 was detected in 25.3 % of dysplastic tissues, with very wide variability between studies indicating prevalence rates 0–100 % [143]. The oral cavity dysplasia sites included in these studies were from the tongue, floor of the mouth/ventral tongue, buccal gingiva/vestibule, hard palate, oral commissure/lip, and other unspecified sites in the oral cavity. While DNA presence alone quantifies prevalence, it does not necessarily correlate with viral transformation. However, in 2011, Syrjanen et al. performed a systematic review of case-control studies published from 1966 to 2010 that have investigated the association of HPV with potentially malignant disorders such as oral lichen planus, leukoplakia, erythroplakia, and oral proliferative verrucous leukoplakia (956 cases and 675 controls) [144]. Similarly, during this timeframe case-control studies investigating HPV's association with oral squamous cell carcinoma (involving the oral cavity only) were also reviewed and summarized (1,885 cases and 2,248 controls). Based on this report, HPV16 appears to be associated with the development of dysplastic oral lesions (oral lichen planus: odds ratio = 5.61; 95 % CI: 2.42–5.99, and leukoplakia: odds ratio = 4.47; 95 % CI: 2.22–8.98) as well as oral cavity cancers (odds ratio = 3.86; 95 % CI: 2.16–6.86) [144]. A similar yet weaker association was also reported in an earlier study conducted in 2006 (odds ratio = 2.1; 95 % CI: 1.2–3.4) [145]. Overall, this suggests that HPV may be associated with at least a subset of oral cavity tumors, but unlike HPV-positive oropharyngeal cancers, a causal association between HPV and survival advantage for patients with HPV-positive oral cavity cancers has not been clearly delineated.

A steady decline in the incidence of nonoropharyngeal cancers overall has been reported in the USA and worldwide for all racial/ethnic groups and genders and may be reflective of the declining prevalence of tobacco and alcohol consumption [146–150]. In contrast, however, more careful analyses have reported an increase in the incidence of oral tongue cancers in younger adults despite the overall decline in tobacco use [151–153]. In the USA, examination of data from the Surveillance, Epidemiology, and End Results Program of the US National Cancer Institute revealed that the incidence of oral tongue cancer was not significantly different between 1973 and 2006 among white males, but has significantly, although modestly increased among white females and declined among African American males and females [154]. Possible suggestions for the increasing incidence of oral tongue cancer among white females might be related to the recent trend of increased smoking prevalence among women, or other unknown environmental factors. A similar trend of increasing incidence between oral tongue and oropharyngeal cancers might suggest similar etiology, such

as HPV. Whether or not oral tongue cancers are attributable to HPV is yet to be confirmed. One argument suggesting this is unlikely is that there does not appear to be a survival advantage among oral tongue cancer patients compared to those diagnosed with cancer in other nonoropharyngeal subsites [155]; and that there is no significant association between *TP53* expression (measured by immunohistochemistry) and HPV infection in oral tongue cancers [156]. Some evidence supports HPV involvement in at least a subset of oral tongue cancers, since immunostaining assays revealed a correlation between HPV16 E6 expression and p53 loss as well as HPV16 E7 expression and pRb loss [157]; therefore, further investigations are needed.

A substantial number of studies have also investigated the carcinogenic role of HPV in laryngeal cancer and recent evidence suggests that HPV16 might be associated with approximately 20 % of these tumors. Among twelve studies conducted through 2012, an association of HPV16 rather than HPV18 was observed (HPV16 pooled odds ratio: 6.07, 95 % CI: 3.44–10.70 vs. HPV18 pooled odds ratio: 4.16, 95 % CI: 0.87–20.04). In summary, while both cutaneous and mucosal HPVs have been detected in the oral cavity and the larynx, the presence of mucosal HPVs, and particularly HPV16 DNA correlated with the presence of viral transcripts and enhanced *CDKN2A* (P16) detected in some studies, seems to suggest a possible etiologic role in a small subset of oral cavity and laryngeal tumors [158–160]**.** However, unlike HPV-positive oropharyngeal cancers, there has been no documented positive impact on treatment outcomes [160–164].

3.6 HPV Variants: Functional Differences and Implications for Risk and Treatment

3.6.1 HPV Intratypic Variants

Within the differing genotypes of HPV, intratypic variants have been reported that may affect the ability of the virus to induce cancer. The nomenclature for HPV has been established by the International Committee on Taxonomy of Viruses (ICTV) and is based on recommendations from the Study Group of Papillomavirus [165–167]. While definitions for genera and species have been defined, there are no set standards defined below the species level. Currently, a distinct HPV genotype is defined as having $> 10\,\%$ nucleotide sequence variation within the L1 gene; in contrast, the nucleotide sequences of intratypic variants typically differ between 2–5 %. Since there are no defined standards, phylogenetic investigations of HPV16 and HPV18 variants have been limited initially to partial URR and E6 sequences, with this more recently augmented with studies of complete HPV genomes. This variability in the classification of HPV variants has resulted in limitations in the ability to compare results from independent studies, since different regions of the viral genomes have been evaluated. Nevertheless, extensive studies have been conducted on HPV16 and to a lesser extent on HPVs 18 and 45 [168–173] which have provided some insight on HPV variant lineages and sublineages.

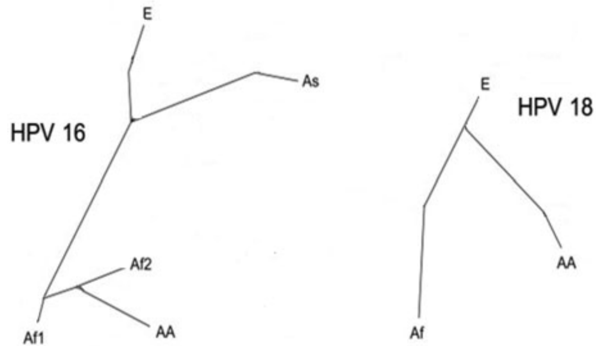

Fig. 3.6 Distinct phylogenetic branches of HPV16 and HP18 variants. The five HPV16 variants include the European (E) prototype, East Asian (As), Asian American (AA) and African (Af1 and Af2) variants. The three HPV 18 variants include the European (E) prototype, Asian American (AA) and African variants (Af)

For any given HPV genotype, related variants tend to cluster geographically as well as within ethnic groups. There are five distinct phylogenetic branches of HPV16 variants (AA, Af1, Af2, As, and E), and three branches of HPV18 variants (A, AA, and E) [169–171] (Fig. 3.6). All HPV16 variants are detected in different populations worldwide but at different frequencies depending on geography and ethnicity. The prototype HPV16 virus was first isolated and sequenced from a German cervical cancer patient [174] and belongs to the European (E) variant lineage. HPV16 E variants are predominantly detected in European populations. There are two African variants (Af1 and Af2) detected primarily in African populations, while the Asian American (AA) variants are primarily detected in Asian and indigenous populations in America. In contrast the East Asian (As) variants are predominant in Chinese and Japanese populations [169], [170]. Similarly for HPV18, the frequencies of these variants also occur according to geography and ethnicity [171], [173]. The prototype HPV18 virus belongs to the Asian American (AA) phylogenetic branch and although identified and sequenced in Germany was isolated from a cervical cancer patient from Northeast Brazil [175]. Unlike HPV16, HPV18 AA variants are found primarily in East Asians and American Indians. African populations tend to carry the A variants and Europeans the E variants.

A mixture of HPV16 and HPV18 variants is often observed in North, Central and South American populations, and is reflective of both the multiethnic groups arising from immigration, and the predilection to maintain distinct ethnic groups well after immigration [169], [171]. Longitudinal studies of cervical HPV infection have shown that women of European descent are more likely to be persistently infected with E variants while African American women are more likely to be persistently infected with Af variants [176]. Pathogenic differences have also been noted between variants. Studies conducted in various geographic regions show that non-European variants are more frequently persistent compared to European variants, and the risk of cancer progression in the cervix and anal mucosa is higher compared to European variants [177–183]. Furthermore, differing risks of high-grade cervical intraepithelial neoplasia (CIN) have been demonstrated by different HPV variants [182], [184]. The risk of developing high-grade CIN was threefold greater for women infected with

non-European HPV16 variants compared to European HPV16 variants (Af2: RR = 2.7, 95 % CI: 1.0–7.0 and AA: RR = 3.1, 95 % CI: 1.6–6.0) and similar observations have been shown for anal carcinoma in situ [181] in HIV-positive men (HPV16 non-European HPV variants: RR = 3.2, 95 % CI: 1.0–10.3).

3.6.2 Functional and Medical Implications of HPV Variants in Cancer

The genomic diversity within the viral coding sequences and regulatory regions of HPV16 and HPV18 variants may result in (1) functional differences in the viral proteins and (2) differences in the level of viral replication and transcription. Current studies have evaluated genetic variations in the HPV16, HPV18 E6, and E7 genes with primary focus on the effects on gene expression and the ability to interact with host proteins.

Variant genomes of HPV16 have been shown to have different activities in degrading p53 protein [185]. For example, the Af2 variant (z84) shows lower activity for p53 degradation compared to the AA variant (512). It has been suggested that an R10I substitution might contribute to the lower activity observed for Af2 z84 and the Q14H and/or S138C substitution(s) might contribute to the higher activity of AA 512. However, since multiple amino acid changes characterize each intratypic variant, it is not sufficient to assume that functional differences between variants might be attributed to a single amino acid change but rather specific combinations of amino acid changes. Variant E6 genomes of HPV18 have also been shown to affect the degradation of p53 protein due to differences in differential splicing in the E6 gene [186]. In this study, nucleotide changes promote alternative splicing in the Af E6 variant resulting in a reduction of functional E6 protein expression. In contrast, the European variant was reported to have higher functional E6 protein expression and thus less alternative splicing in the E6 gene [186]. Other genetic variations in HPV E6 and E7 have been shown to have differential effects on cell protein interactions involving mitogen-activated protein kinase (MAPK) signaling [187] and protein kinase B/phosphatidylinositol 3-kinase (AKT/PI3K) signaling [188] which are involved in cell survival and proliferation. Genetic variations in the E2 gene as well as the URR have also been shown to affect E6 expression [189–193], and thus viral oncogenicity. In addition, sequence variations in the URR which affect the binding sites for the transcription factors AP-1, NFI, Oct-1, TEF-1, and YY1, have been documented and shown to result in differences in HPV replication rates [194] as well as transcriptional activation of the E6/E7 promoter [190–193].

To date, there is little that is known about the clinical impact and carcinogenic relevance of HPV variants in oropharyngeal cancer. A recent study compared European HPV16 variants between 108 tonsillar squamous cell carcinoma and 52 cervical cancer samples collected from patients diagnosed between 2000 and 2008 [195]. One European HPV16 variant has an R10G change in the E6 gene, and a higher frequency of this variant was reported in tonsillar cancers compared to cervical cancer samples

(19 vs. 4 %) [195]. Another study of squamous cell carcinomas arising in the upper aerodigestive tract shows the predominance of the HPV16 L83V variant (i.e., 5/8, 63 % HPV-positive cases) but the prognostic significance is yet to be determined [196]. While the relevance of this difference is not currently apparent, future studies of HPV intratypic variants and their functional significance as well as their impact on the natural history and treatment for oropharyngeal cancers are warranted.

In summary, HPV-positive oropharyngeal tumors represent a distinct clinicopathological profile, but there are still many unanswered questions. The mechanisms surrounding the survival advantage for patients with these tumors need further study. In addition, the significance of and mechanism related to high-risk HPV variants and disease development as well as prognosis are currently poorly understood. Further studies should address the biology of intratypic HPV variants in oropharyngeal cancers, and identify additional useful clinical markers to enable appropriate risk stratification, with the goal of optimizing patient treatment.

References

1. zur Hausen H, de Villiers EM. Human papillomaviruses. Ann Rev Microbiol. 1994;48:427–47.
2. Syrjanen K, Syrjanen S, Lamberg M, Pyrhonen S, Nuutinen J. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Surg. 1983;12:418–24.
3. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92:709–20.
4. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev. 2005;14:467–75.
5. Shindoh M, Chiba I, Yasuda M, Saito T, Funaoka K, Kohgo T, Amemiya A, Sawada Y, Fujinaga K. Detection of human papillomavirus DNA sequences in oral squamous cell carcinomas and their relation to p53 and proliferating cell nuclear antigen expression. Cancer. 1995;76:1513–21.
6. Haraf DJ, Nodzenski E, Brachman D, Mick R, Montag A, Graves D, Vokes EE, Weichselbaum RR. Human papilloma virus and p53 in head and neck cancer: clinical correlates and survival. Clin Cancer Res. 1996;2:755–62.
7. Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP. Human papillomavirus (HPV) in head and neck cancer. An association of HPV 16 with squamous cell carcinoma of Waldeyer's tonsillar ring. Cancer. 1997;79:595–604.
8. Mineta H, Ogino T, Amano HM, Ohkawa Y, Araki K, Takebayashi S, Miura K. Human papilloma virus (HPV) type 16 and 18 detected in head and neck squamous cell carcinoma. Anticancer Res. 1998;18:4765–8.
9. Adams V, Schmid S, Zariwala M, Schmid M, Kleihues P, Briner J, Schafer R. Prevalence of human papilloma virus DNA in head and neck cancers carrying wild-type or mutant p53 tumor suppressor genes. Anticancer Res. 1999;19:1–6.
10. Nishioka S, Fukushima K, Nishizaki K, Gunduz M, Tominaga S, Fukazawa M, Monden N, Watanabe S, Masuda Y, Ogura H. Human papillomavirus as a risk factor for head and neck cancers—a case-control study. Acta Otolaryngol Suppl. 1999;540:77–80.

11. Badaracco G, Venuti A, Morello R, Muller A, Marcante ML. Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res. 2000;20:1301–5.
12. Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E. Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. Int J Cancer. 2000;89:300–4.
13. Sisk EA, Bradford CR, Jacob A, Yian CH, Staton KM, Tang G, Harris MO, Carey TE, Lancaster WD, Gregoire L. Human papillomavirus infection in "young" versus "old" patients with squamous cell carcinoma of the head and neck. Head Neck. 2000;22:649–57.
14. Venuti A, Manni V, Morello R, De Marco F, Marzetti F, Marcante ML. Physical state and expression of human papillomavirus in laryngeal carcinoma and surrounding normal mucosa. J Med Virol. 2000;60:396–402.
15. Klussmann JP, Weissenborn SJ, Wieland U, Dries V, Kolligs J, Jungehuelsing M, Eckel HE, Dienes HP, Pfister HJ, Fuchs PG. Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. Cancer. 2001;92:2875–84.
16. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM. Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer. 2001;92:805–13.
17. Miller CS, Johnstone BM. Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982–1997. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001;91:622–35.
18. Mork J, Lie AK, Glattre E, Hallmans G, Jellum E, Koskela P, Moller B, Pukkala E, Schiller JT, Youngman L, Lehtinen M, Dillner J. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med. 2001;344:1125–31.
19. Hodge KM, Flynn MB, Drury T. Squamous cell carcinoma of the upper aerodigestive tract in nonusers of tobacco. Cancer. 1985;55:1232–5.
20. Hoshikawa T, Nakajima T, Uhara H, Gotoh M, Shimosato Y, Tsutsumi K, Ono I, and Ebihara S Detection of human papillomavirus DNA in laryngeal squamous cell carcinomas by polymerase chain reaction. Laryngoscope. 1990;100:647–50.
21. Niedobitek G, Pitteroff S, Herbst H, Shepherd P, Finn T, Anagnostopoulos I, Stein H. Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil. J Clin Pathol. 1990;43:918–21.
22. Blot WJ, Devesa SS, McLaughlin JK, Fraumeni JF Jr. Oral and pharyngeal cancers. Cancer Surv. 1994;19–20:23–42.
23. Ostwald C, Muller P, Barten M, Rutsatz K, Sonnenburg M, Milde-Langosch K, Loning T. Human papillomavirus DNA in oral squamous cell carcinomas and normal mucosa. J Oral Pathol Med. 1994;23:220–5.
24. Sethi S, Ali-Fehmi R, Franceschi S, Struijk L, Van Doorn LJ, Quint W, Albashiti B, Ibrahim M, Kato I. Characteristics and survival of head and neck cancer by HPV status: a cancer registry-based study. Int J Cancer. 2012;131:1179–86.
25. Burk RD, Chen Z, van Doorslaer K. Human papillomaviruses: genetic basis of carcinogenicity. Public Health Genomics. 2009;12:281–90.
26. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc Natl Acad Sci U.S.A. 2009;106:20458–63.
27. Schiller JT, Day PM, Kines RC. Current understanding of the mechanism of HPV infection. Gynecol Oncol. 2010;118:S12–17.
28. McBride AA, Oliveira JG, McPhillips MG. Partitioning viral genomes in mitosis: same idea, different targets. Cell Cycle. 2006;5:1499–502.
29. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K. Mechanisms of human papillomavirus-induced oncogenesis. J Virol. 2004;78:11451–60.
30. Ma T, Zou N, Lin BY, Chow LT, Harper JW. Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication. Proc Natl Acad Sci U.S.A. 1999;96:382–7.

31. Liu JS, Kuo SR, Makhov AM, Cyr DM, Griffith JD, Broker TR, Chow LT. Human Hsp70 and Hsp40 chaperone proteins facilitate human papillomavirus-11 E1 protein binding to the origin and stimulate cell-free DNA replication. J Biol Chem. 1998;273:30704–12.
32. Lee D, Sohn H, Kalpana GV, Choe J. Interaction of E1 and hSNF5 proteins stimulates replication of human papillomavirus DNA. Nature. 1999;399:487–91.
33. Swindle CS, Engler JA. Association of the human papillomavirus type 11 E1 protein with histone H1. J Virol. 1998;72:1994–2001.
34. Yasugi T, Vidal M, Sakai H, Howley PM, Benson JD. Two classes of human papillomavirus type 16 E1 mutants suggest pleiotropic conformational constraints affecting E1 multimerization, E2 interaction, and interaction with cellular proteins. J Virol. 1997;71:5942–51.
35. Lin BY, Makhov AM, Griffith JD, Broker TR, Chow LT. Chaperone proteins abrogate inhibition of the human papillomavirus (HPV) E1 replicative helicase by the HPV E2 protein. Mol Cell Biol. 2002;22:6592–604.
36. Zobel T, Iftner T, Stubenrauch F. The papillomavirus E8-E2C protein represses DNA replication from extrachromosomal origins. Mol Cell Biol. 2003;23:8352–62.
37. Stubenrauch F, Zobel T, Iftner T. The E8 domain confers a novel long-distance transcriptional repression activity on the E8E2C protein of high-risk human papillomavirus type 31. J Virol. 2001;75:4139–49.
38. Fertey J, Hurst J, Straub E, Schenker A, Iftner T, Stubenrauch F. Growth inhibition of HeLa cells is a conserved feature of high-risk human papillomavirus E8^E2C proteins and can also be achieved by an artificial repressor protein. J Virol. 2011;85:2918–26.
39. Doorbar J, Ely S, Sterling J, McLean C, Crawford L. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature. 1991;352:824–7.
40. Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P, Omary MB, Doorbar J. Functional analysis of the human papillomavirus type 16 E1 = E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. J Virol. 2004;78:821–33.
41. Conrad M, Bubb VJ, Schlegel R. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. J Virol. 1993;67:6170–8.
42. Oetke C, Auvinen E, Pawlita M, Alonso A. Human papillomavirus type 16 E5 protein localizes to the Golgi apparatus but does not grossly affect cellular glycosylation. Arch Virol. 2000;145:2183–91.
43. DiMaio D, Mattoon D. Mechanisms of cell transformation by papillomavirus E5 proteins. Oncogene. 2001;20:7866–73.
44. Hafner N, Driesch C, Gajda M, Jansen L, Kirchmayr R, Runnebaum IB, Durst M. Integration of the HPV16 genome does not invariably result in high levels of viral oncogene transcripts. Oncogene. 2008;27:1610–7.
45. Bouvard V, Matlashewski G, Gu ZM, Storey A, Banks L. The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. Virology. 1994;203:73–80.
46. Chang JL, Tsao YP, Liu DW, Huang SJ, Lee WH, Chen SL. The expression of HPV-16 E5 protein in squamous neoplastic changes in the uterine cervix. J Biomed Sci. 2001;8:206–13.
47. Ashrafi GH, Brown DR, Fife KH, Campo MS. Down-regulation of MHC class I is a property common to papillomavirus E5 proteins. Virus Res. 2006;120:208–11.
48. Zhang B, Li P, Wang E, Brahmi Z, Dunn KW, Blum JS, Roman A. The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. Virology. 2003;310:100–8.
49. Massimi P, Shai A, Lambert P, Banks L. HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background. Oncogene. 2008;27:1800–4.
50. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell. 1993;75:495–505.

51. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science. 1990;248:76–9.
52. Gewin L, Myers H, Kiyono T, Galloway DA. Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. Genes Dev. 2004;18:2269–82.
53. Ronco LV, Karpova AY, Vidal M, Howley PM. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev. 1998;12:2061–72.
54. Tong X, Howley PM. The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. Proc Natl Acad Sci U.S.A. 1997;94:4412–7.
55. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res. 1996;56:4620–4.
56. Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, Howley PM. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proc Natl Acad Sci U.S.A. 1996;93:4350–4.
57. Dyson N, Guida P, Munger K, Harlow E. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J Virol. 1992;66:6893–902.
58. Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 1989;8:4099–105.
59. Phelps WC, Bagchi S, Barnes JA, Raychaudhuri P, Kraus V, Munger K, Howley PM, Nevins JR. Analysis of trans activation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. J Virol. 1991;65:6922–30.
60. Arroyo M, Bagchi S, Raychaudhuri P. Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. Mol Cell Biol. 1993;13:6537–46.
61. McIntyre MC, Ruesch MN, Laimins LA. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology. 1996;215:73–82.
62. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol. 2002;55:244–65.
63. Bosch FX, de Sanjose S. Chapter 1: Human papillomavirus and cervical cancer–burden and assessment of causality. J Natl Cancer Inst Monogr. 2003;3–13.
64. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst. 2000;92:690–8.
65. Ragin CC R, Reshmi SC, Gollin SM. Mapping and analysis of HPV16 integration sites in a head and neck cancer cell line. Int J Cancer. 2004;110:701–09.
66. Wentzensen N, Vinokurova S, von Knebel Doeberitz M. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. Cancer Res. 2004;64:3878–84.
67. Mellin H, Dahlgren L, Munck-Wikland E, Lindholm J, Rabbani H, Kalantari M, Dalianis T. Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer. Int J Cancer. 2002;102:152–8.
68. Deng Z, Hasegawa M, Kiyuna A, Matayoshi S, Uehara T, Agena S, Yamashita Y, Ogawa K, Maeda H, Suzuki M. Viral load, physical status, and E6/E7 mRNA expression of human papillomavirus in head and neck squamous cell carcinoma. Head Neck. 2013;35:800–8.
69. Badaracco G, Venuti A, Morello R, Muller A, Marcante ML. Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res. 2000;20:1301–5.
70. Badaracco G, Rizzo C, Mafera B, Pichi B, Giannarelli D, Rahimi SS, Vigili MG, Venuti A. Molecular analyses and prognostic relevance of HPV in head and neck tumours. Oncol Rep. 2007;17:931–9.
71. Koskinen WJ, Chen RW, Leivo I, Makitie A, Back L, Kontio R, Suuronen R, Lindqvist C, Auvinen E, Molijn A, Quint WG, Vaheri A, Aaltonen LM. Prevalence and physical status

of human papillomavirus in squamous cell carcinomas of the head and neck. Int J Cancer. 2003;107:401–6.

72. Watts SL, Brewer EE, Fry TL. Human papillomavirus DNA types in squamous cell carcinomas of the head and neck. Oral Surg Oral Med Oral Pathol. 1991;71:701–7.
73. Markowitz LE, Sternberg M, Dunne EF, McQuillan G, Unger ER. Seroprevalence of human papillomavirus types 6, 11, 16, and 18 in the United States: National Health and Nutrition Examination Survey 2003–2004. J Infect Dis. 2009;200:1059–67.
74. Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L, Graubard BI, Chaturvedi AK. Prevalence of oral HPV infection in the United States, 2009–2010. JAMA. 2012;307: 693–703.
75. Safaeian M, Porras C, Schiffman M, Rodriguez AC, Wacholder S, Gonzalez P, Quint W, Van Doorn LJ, Sherman ME, Xhenseval V, Herrero R, Hildesheim A. Epidemiological study of anti-HPV16/18 seropositivity and subsequent risk of HPV16 and -18 infections. J Natl Cancer Inst. 2010;102:1653–62.
76. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. 2010. Lyon, France: International Agency for Research on Cancer; 2010. (Ref Type: Generic).
77. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin K. SEER Cancer Statistics Review, 1975–2009.Bethesda, MD, based on November 2011 SEER data submission, posted to the SEER web site, April 2012: National Cancer Institute, 2012.
78. Maritz GS, Mutemwa M. Tobacco smoking: patterns, health consequences for adults, and the long-term health of the offspring. Glob J Health Sci. 2012;4:62–75.
79. Roberts JC, Li G, Reitzel LR, Wei Q, Sturgis EM. No evidence of sex-related survival disparities among head and neck cancer patients receiving similar multidisciplinary care: a matched-pair analysis. Clin Cancer Res. 2010;16:5019–27.
80. Znaor T, Vucemilo L, Kulis T, Znaor A. Incidence and mortality trends of head and neck cancer in Croatia in the period 1988–2008. Acta Otolaryngol. 2013;133(3):305–12.
81. Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D, Ramqvist T, Lindholm J, Sparen P, Ye W, Dahlstrand H, Munck-Wikland E, Dalianis T. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int J Cancer. 2009;125:362–6.
82. Gillison ML, Koch WM, Shah KV. Human papillomavirus in head and neck squamous cell carcinoma: are some head and neck cancers a sexually transmitted disease? Curr Opin Oncol. 1999;11:191–9.
83. Braakhuis BJ, Snijders PJ, Keune WJ, Meijer CJ, Ruijter-Schippers HJ, Leemans CR, Brakenhoff RH. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. J Natl Cancer Inst. 2004;96:998–1006.
84. van Houten VM, Snijders PJ, van den Brekel MW, Kummer JA, Meijer CJ, van Leeuwen B, Denkers F, Smeele LE, Snow GB, Brakenhoff RH. Biological evidence that human papillomaviruses are etiologically involved in a subgroup of head and neck squamous cell carcinomas. Int J Cancer. 2001;93:232–5.
85. Wiest T, Schwarz E, Enders C, Flechtenmacher C, Bosch FX. Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. Oncogene. 2002;21:1510–7.
86. McGorray SP, Guo Y, Logan H. Trends in incidence of oral and pharyngeal carcinoma in Florida: 1981–2008. J Public Health Dent. 2012;72:68–74.
87. Golas SM. Trends in palatine tonsillar cancer incidence and mortality rates in the United States. Community Dent Oral Epidemiol. 2007;35:98–108.
88. Forte T, Niu J, Lockwood GA, Bryant HE. Incidence trends in head and neck cancers and human papillomavirus (HPV)-associated oropharyngeal cancer in Canada, 1992–2009. Cancer Causes Control. 2012;23:1343–8.

89. de Souza DL, de Camargo CM, Perez MM, Curado MP. Trends in the incidence of oral cavity and oropharyngeal cancers in Spain. Head Neck. 2012;34:649–54.
90. Attner P, Du J, Nasman A, Hammarstedt L, Ramqvist T, Lindholm J, Marklund L, Dalianis T, Munck-Wikland E. The role of human papillomavirus in the increased incidence of base of tongue cancer. Int J Cancer. 2010;126:2879–84.
91. Ernster JA, Sciotto CG, O'Brien MM, Finch JL, Robinson LJ, Willson T, Mathews M. Rising incidence of oropharyngeal cancer and the role of oncogenic human papilloma virus. Laryngoscope. 2007;117:2115–28.
92. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29:4294–301.
93. D'Souza G, Zhang HH, D'Souza WD, Meyer RR, Gillison ML. Moderate predictive value of demographic and behavioral characteristics for a diagnosis of HPV16-positive and HPV16-negative head and neck cancer. Oral Oncol. 2010;46:100–4.
94. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008;100:261–9.
95. Kingma DW, Allen RA, Moore W, Caughron SK, Melby M, Gillies EM, Marlar RA, Dunn ST. HPV genotype distribution in oral and oropharyngeal squamous cell carcinoma using seven in vitro amplification assays. Anticancer Res. 2010;30:5099–104.
96. Mehanna H, Beech T, Nicholson T, El Hariry I, McConkey C, Paleri V, Roberts S. Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer-systematic review and meta-analysis of trends by time and region. Head Neck. 2013;35:747–55.
97. Brown LM, Check DP, Devesa SS. Oral cavity and pharynx cancer incidence trends by subsite in the United States: changing gender patterns. J Oncol. 2012;2012:649498.
98. El Mofty SK, Lu DW. Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: a distinct clinicopathologic and molecular disease entity. Am J Surg Pathol. 2003;27:1463–70.
99. Cole L, Polfus L, Peters ES. Examining the incidence of human papillomavirus-associated head and neck cancers by race and ethnicity in the U.S., 1995–2005. PLoS One. 2012;7:e32657.
100. Brandsma JL, Abramson AL. Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg. 1989;115:621–5.
101. Niedobitek G, Pitteroff S, Herbst H, Shepherd P, Finn T, Anagnostopoulos I, Stein H. Detection of human papillomavirus type 16 DNA in carcinomas of the palatine tonsil. J Clin Pathol. 1990;43:918–21.
102. Snijders PJ, Cromme FV, van den Brule AJ, Schrijnemakers HF, Snow GB, Meijer CJ, Walboomers JM. Prevalence and expression of human papillomavirus in tonsillar carcinomas, indicating a possible viral etiology. Int J Cancer. 1992;51:845–50.
103. Smith EM, Pignatari SS, Gray SD, Haugen TH, Turek LP. Human papillomavirus infection in papillomas and nondiseased respiratory sites of patients with recurrent respiratory papillomatosis using the polymerase chain reaction. Arch Otolaryngol Head Neck Surg. 1993;119:554–7.
104. Watanabe S, Ogura H, Fukushima K, Yabe Y. Comparison of Virapap filter hybridization with polymerase chain reaction and Southern blot hybridization methods for detection of human papillomavirus in tonsillar and pharyngeal cancers. Eur Arch Otorhinolaryngol. 1993;250:115–9.
105. Fukushima K, Ogura H, Watanabe S, Yabe Y, Masuda Y. Human papillomavirus type 16 DNA detected by the polymerase chain reaction in non-cancer tissues of the head and neck. Eur Arch Otorhinolaryngol. 1994;251:109–12.
106. Tominaga S, Fukushima K, Nishizaki K, Watanabe S, Masuda Y, Ogura H. Presence of human papillomavirus type 6f in tonsillar condyloma acuminatum and clinically normal tonsillar mucosa. Jpn J Clin Oncol. 1996;26:393–7.

107. Strome SE, Savva A, Brissett AE, Gostout BS, Lewis J, Clayton AC, McGovern R, Weaver AL, Persing D, Kasperbauer JL. Squamous cell carcinoma of the tonsils: a molecular analysis of HPV associations. Clin Cancer Res. 2002;8:1093–100.
108. Mammas IN, Sourvinos G, Michael C, Spandidos DA. Human papilloma virus in hyperplastic tonsillar and adenoid tissues in children. Pediatr Infect Dis J. 2006;25:1158–62.
109. Sisk J, Schweinfurth JM, Wang XT, Chong K. Presence of human papillomavirus DNA in tonsillectomy specimens. Laryngoscope. 2006;116:1372–4.
110. Chen R, Sehr P, Waterboer T, Leivo I, Pawlita M, Vaheri A, Aaltonen LM. Presence of DNA of human papillomavirus 16 but no other types in tumor-free tonsillar tissue. J Clin Microbiol. 2005;43:1408–10.
111. Duray A, Descamps G, Bettonville M, Sirtaine N, Ernoux-Neufcoeur P, Guenin S, Mouallif M, Depuydt CE, Delvenne P, Saussez S. High prevalence of high-risk human papillomavirus in palatine tonsils from healthy children and adults. Otolaryngol Head Neck Surg. 2011;145:230–5.
112. do Sacramento PR, Babeto E, Colombo J, Cabral Ruback MJ, Bonilha JL, Fernandes AM, Pereira Sobrinho JS, de Souza FP, Villa LL, Rahal P. The prevalence of human papillomavirus in the oropharynx in healthy individuals in a Brazilian population. J Med Virol. 2006;78: 614–8.
113. Ernster JA, Sciotto CG, O'Brien MM, Robinson LJ, Willson T. Prevalence of oncogenic human papillomavirus 16 and 18 in the palatine tonsils of the general adult population. Arch Otolaryngol Head Neck Surg. 2009;135:554–7.
114. Fouret P, Martin F, Flahault A, Saint-Guily JL. Human papillomavirus infection in the malignant and premalignant head and neck epithelium. Diagn Mol Pathol. 1995;4:122–7.
115. Sikora AG, Morris LG, Sturgis EM. Bidirectional association of anogenital and oral cavity/pharyngeal carcinomas in men. Arch Otolaryngol Head Neck Surg. 2009;135:402–5.
116. Frisch M, Biggar RJ. Aetiological parallel between tonsillar and anogenital squamous-cell carcinomas. Lancet. 1999;354:1442–43.
117. Rose Ragin CC, Taioli E. Second primary head and neck tumor risk in cervical cancer patients—SEER data analysis. Head Neck. 2008;30:58–66.
118. Crawford R, Grignon AL, Kitson S, Winder DM, Ball SL, Vaughan K, Stanley MA, Sterling JC, Goon PK. High prevalence of HPV in non-cervical sites of women with abnormal cervical cytology. BMC Cancer. 2011;11:473.
119. Hemminki K, Dong C, Frisch M. Tonsillar and other upper aerodigestive tract cancers among cervical cancer patients and their husbands. Eur J Cancer Prev. 2000;9:433–7.
120. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med. 2007;356:1944–56.
121. D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison ML. Oral sexual behaviors associated with prevalent oral human papillomavirus infection. J Infect Dis. 2009;199:1263–9.
122. Pickard RK, Xiao W, Broutian TR, He X, Gillison ML. The prevalence and incidence of oral human papillomavirus infection among young men and women, aged 18–30 years. Sex Transm Dis. 2012;39:559–66.
123. Dahlstrom KR, Li G, Tortolero-Luna G, Wei Q, Sturgis EM. Differences in history of sexual behavior between patients with oropharyngeal squamous cell carcinoma and patients with squamous cell carcinoma at other head and neck sites. Head Neck. 2011;33:847–55.
124. Heck JE, Berthiller J, Vaccarella S, Winn DM, Smith EM, Shan'gina, O., Schwartz SM, Purdue MP, Pilarska A, Eluf-Neto J, Menezes A, McClean MD, Matos E, Koifman S, Kelsey KT, Herrero R, Hayes RB, Franceschi S, Wunsch-Filho V, Fernandez L, Daudt AW, Curado MP, Chen C, Castellsague X, Ferro G, Brennan P, Boffetta P, Hashibe M. Sexual behaviours and the risk of head and neck cancers: a pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. Int J Epidemiol. 2010;39:166–81.
125. Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ, Solomon B, Choi J, O'Sullivan B, Kenny LM, McArthur GA. Prognostic significance of p16INK4A and human papillomavirus

in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol. 2010;28:4142–48.

126. Young RJ, Rischin D, Fisher R, McArthur GA, Fox SB, Peters LJ, Corry J, Lim A, Waldeck K, Solomon B. Relationship between epidermal growth factor receptor status, p16(INK4A), and outcome in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2011;20:1230–7.
127. Hoff CM, Lassen P, Eriksen JG, Hansen HS, Specht L, Overgaard M, Grau C, Johansen J, Bentzen J, Andersen L, Evensen JF, Overgaard J. Does transfusion improve the outcome for HNSCC patients treated with radiotherapy? – results from the randomized DAHANCA 5 and 7 trials. Acta Oncol. 2011;50:1006–14.
128. Jo S, Juhasz A, Zhang K, Ruel C, Loera S, Wilczynski SP, Yen Y, Liu X, Ellenhorn J, Lim D, Paz B, Somlo G, Vora N, Shibata S. Human papillomavirus infection as a prognostic factor in oropharyngeal squamous cell carcinomas treated in a prospective phase II clinical trial. Anticancer Res. 2009;29:1467–74.
129. Kies MS, Holsinger FC, Lee JJ, William WN, Jr., Glisson BS, Lin HY, Lewin JS, Ginsberg LE, Gillaspy KA, Massarelli E, Byers L, Lippman SM, Hong WK, el Naggar AK, Garden AS, Papadimitrakopoulou V. Induction chemotherapy and cetuximab for locally advanced squamous cell carcinoma of the head and neck: results from a phase II prospective trial. J Clin Oncol. 2010;28:8–14.
130. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, Overgaard J. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J Clin Oncol. 2009;27:1992–8.
131. Posner MR, Lorch JH, Goloubeva O, Tan M, Schumaker LM, Sarlis NJ, Haddad RI, Cullen KJ. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. Ann Oncol. 2011;22:1071–7.
132. Quon H, Cohen MA, Montone KT, Ziober A, Wang LP, Weinstein GS, O'Malley BW Jr. Transoral robotic surgery and adjuvant therapy for oropharyngeal carcinomas and the influence of p16(INK4a)on treatment outcomes. Laryngoscope. 2013;123(3):635–40.
133. Ragin CC, Taioli E, Weissfeld JL, White JS, Rossie KM, Modugno F, Gollin SM. 11q13 amplification status and human papillomavirus in relation to p16 expression defines two distinct etiologies of head and neck tumours. Br J Cancer. 2006;95:1432–8.
134. Smeets SJ, Braakhuis BJ, Abbas S, Snijders PJ, Ylstra B, van de Wiel MA, Meijer GA, Leemans CR, Brakenhoff RH. Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. Oncogene. 2006;25:2558–64.
135. Schlecht NF, Burk RD, Adrien L, Dunne A, Kawachi N, Sarta C, Chen Q, Brandwein-Gensler M, Prystowsky MB, Childs G, Smith RV, Belbin TJ. Gene expression profiles in HPV-infected head and neck cancer. J Pathol. 2007;213:283–93.
136. Slebos RJ, Yi Y, Ely K, Carter J, Evjen A, Zhang X, Shyr Y, Murphy BM, Cmelak AJ, Burkey BB, Netterville JL, Levy S, Yarbrough WG, Chung CH. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. Clin Cancer Res. 2006;12:701–9.
137. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363:24–35.
138. Granata R, Miceli R, Orlandi E, Perrone F, Cortelazzi B, Franceschini M, Locati LD, Bossi P, Bergamini C, Mirabile A, Mariani L, Olmi P, Scaramellini G, Potepan P, Quattrone P, Ang KK, Licitra L. Tumor stage, human papillomavirus and smoking status affect the survival of patients with oropharyngeal cancer:an Italian validation study. Ann Oncol. 2012;23:1832–7.
139. Settle K, Posner MR, Schumaker LM, Tan M, Suntharalingam M, Goloubeva O, Strome SE, Haddad RI, Patel SS, Cambell EV, III, Sarlis N, Lorch J, Cullen KJ. Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal cancer patients. Cancer Prev Res (Phila Pa). 2009;2:776–81.

140. Chernock RD, Zhang Q, El Mofty SK, Thorstad WL, Lewis JS Jr. Human papillomavirus-related squamous cell carcinoma of the oropharynx: a comparative study in whites and African Americans. Arch Otolaryngol Head Neck Surg. 2011;137:163–9.
141. Worsham MJ, Stephen JK, Chen KM, Mahan M, Schweitzer V, Havard S, et al. Improved survival with HPV among African Americans with oropharyngeal cancer. Clin Cancer Res 2013;19(9):2486–92.
142. Worsham MJ, Stephen JK, Chen KM, Mahan M, Havard S, Divine G. Improved Survival with HPV Among African Americans with Oropharyngeal Cancer. 8th International Conference in Head and Neck Cancer; 2012.
143. Jayaprakash V, Reid M, Hatton E, Merzianu M, Rigual N, Marshall J, Gill S, Frustino J, Wilding G, Loree T, Popat S, Sullivan M. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985–2010. Oral Oncol. 2011;47:1048–54.
144. Syrjanen S, Lodi G, von, B., I, Aliko A, Arduino P, Campisi G, Challacombe S, Ficarra G, Flaitz C, Zhou HM, Maeda H, Miller C, Jontell M. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. Oral Dis. 2011;17(Suppl 1):58–72.
145. Hobbs CG, Sterne JA, Bailey M, Heyderman RS, Birchall MA, Thomas SJ. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. Clin Otolaryngol. 2006;31:259–66.
146. Sturgis EM, Cinciripini PM. Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer. 2007;110:1429–35.
147. Brown LM. Epidemiology of alcohol-associated cancers. Alcohol. 2005;35:161–8.
148. Trends in Tobacco Use. American Lung Association Research and Program Services, Epidemiology and Statistics Unit, Epidemiology and Statistics Unit Assessed. 2012. (Ref Type: Electronic Citation).
149. LaVallee RA, Yi, H-Y. Apparent per capita alcohol consumption: national, state, and regional trends, 1977–2009. Surveillance Report #92, National Institute on Alcohol Abuse and Alcoholism Division of Epidemiology and Prevention Research Alcohol Epidemiologic Data System. 2012.
150. US Department of Health and Human Services. Preventing tobacco use among young people: a report of the surgeon general. 1994. Atlanta, Ga, S Dept of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking on Health.
151. Shiboski CH, Schmidt BL, Jordan RC. Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20–44 years. Cancer. 2005;103:1843–9.
152. Muller S, Pan Y, Li R, Chi AC. Changing trends in oral squamous cell carcinoma with particular reference to young patients: 1971–2006. The Emory University experience. Head Neck Pathol. 2008;2:60–6.
153. Schantz SP, Yu GP. Head and neck cancer incidence trends in young Americans, 1973–1997, with a special analysis for tongue cancer. Arch Otolaryngol Head Neck Surg. 2002;128: 268–74.
154. Saba NF, Goodman M, Ward K, Flowers C, Ramalingam S, Owonikoko T, Chen A, Grist W, Wadsworth T, Beitler JJ, Khuri FR, Shin DM. Gender and ethnic disparities in incidence and survival of squamous cell carcinoma of the oral tongue, base of tongue, and tonsils: a surveillance, epidemiology and end results program-based analysis. Oncology. 2011;81: 12–20.
155. Fast Stats: An interactive tool for access to SEER cancer statistics. 2013. Surveillance Research Program, National Cancer Institute. http://seer.cancer.gov/faststats. Accessed: 16. Jan. 2013. (Ref Type: Generic).
156. Seraj JM, Yazdani N, Ashtiani ZO, Seraj SM, Hasheminasab SM, Memar B, Mirashrafi F, Borghei H, Yazdani J, Mostaan LV. TP53 gene expression in HPV-positive oral tongue SCC and its correlation with nodal metastasis. Pathol Res Pract. 2011;207:758–61.

157. Elango KJ, Suresh A, Erode EM, Subhadradevi L, Ravindran HK, Iyer SK, Iyer SK, Kuriakose MA. Role of human papilloma virus in oral tongue squamous cell carcinoma. Asian Pac J Cancer Prev. 2011;12:889–96.
158. Lingen MW, Xiao W, Schmitt A, Jiang B, Pickard R, Kreinbrink P, Perez-Ordonez B, Jordan RC, Gillison ML. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. Oral Oncol. 2013;49(1):1–8.
159. Duray A, Descamps G, Decaestecker C, Remmelink M, Sirtaine N, Lechien J, Ernoux-Neufcoeur P, Bletard N, Somja J, Depuydt CE, Delvenne P, Saussez S. Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. Laryngoscope. 2012;122:1558–65.
160. Isayeva T, Li Y, Maswahu D, Brandwein-Gensler M. Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. Head Neck Pathol. 2012;6(Suppl 1):S104–120.
161. Li X, Li H, Gao J, Yang Y, Zhou F, Gao C, Li M, Jin Q, Gao L. Human Papillomavirus Infection and Laryngeal Cancer Risk: a systematic review and meta-analysis. J Infect Dis. 2013;207:479–88.
162. Wilson DD, Rahimi AS, Saylor DK, Stelow EB, Jameson MJ, Shonka DC, Reibel JF, Levine PA, Read PW. p16 not a prognostic marker for hypopharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2012;138:556–61.
163. Stephen JK, Chen KM, Shah V, Havard S, Lu M, Schweitzer VP, Gardner G, Worsham MJ. Human papillomavirus outcomes in an access-to-care laryngeal cancer cohort. Otolaryngol Head Neck Surg. 2012;146:730–8.
164. Duray A, Descamps G, Arafa M, Decaestecker C, Remmelink M, Sirtaine N, Ernoux-Neufcoeur P, Mutijima E, Somja J, Depuydt CE, Delvenne P, Saussez S. High incidence of high-risk HPV in benign and malignant lesions of the larynx. Int J Oncol. 2011;39:51–9.
165. Bernard HU, Burk RD, Chen Z, van Doorslaer K, Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010;401:70–9.
166. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur HH. Classification of papillomaviruses. Virology. 2004;324:17–27.
167. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Family Papillomaviridae. Virus taxonomy. The Eight Report of the International Committee on Taxonomy of Viruses. 239–255. Elsevier;2005.
168. Chen Z, Terai M, Fu L, Herrero R, Desalle R, Burk RD. Diversifying selection in human papillomavirus type 16 lineages based on complete genome analyses. J Virol. 2005;79: 7014–23.
169. Ho L, Chan SY, Burk RD, Das BC, Fujinaga K, Icenogle JP, Kahn T, Kiviat N, Lancaster W, Mavromara-Nazos P, and. The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. J Virol. 1993;67:6413–23.
170. Yamada T, Manos MM, Peto J, Greer CE, Munoz N, Bosch FX, Wheeler CM. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J Virol. 1997;71:2463–72.
171. Ong CK, Chan SY, Campo MS, Fujinaga K, Mavromara-Nazos P, Labropoulou V, Pfister H, Tay SK, ter Meulen J, Villa LL, and. Evolution of human papillomavirus type 18: an ancient phylogenetic root in Africa and intratype diversity reflect coevolution with human ethnic groups. J Virol., 67:6424–6431, 1993.
172. Chen Z, Schiffman M, Herrero R, Desalle R, Anastos K, Segondy M, Sahasrabuddhe VV, Gravitt PE, Hsing AW, Burk RD. Evolution and taxonomic classification of human papillomavirus 16 (HPV16)-related variant genomes: HPV31, HPV33, HPV35, HPV52, HPV58 and HPV67. PLoS One. 2011;6:e20183.
173. Chen Z, Desalle R, Schiffman M, Herrero R, Burk RD. Evolutionary dynamics of variant genomes of human papillomavirus types 18, 45, and 97. J Virol. 2009;83:1443–55.

174. Durst M, Gissmann L, Ikenberg H, zur HH. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U.S.A. 1983;80:3812–5.
175. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur HH. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. 1984;3:1151–7.
176. Xi LF, Carter JJ, Galloway DA, Kuypers J, Hughes JP, Lee SK, Adam DE, Kiviat NB, Koutsky LA. Acquisition and natural history of human papillomavirus type 16 variant infection among a cohort of female university students. Cancer Epidemiol Biomarkers Prev. 2002;11:343–51.
177. Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T, Franco EL. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. J Gen Virol. 2000;81:2959–68.
178. Hildesheim A, Schiffman M, Bromley C, Wacholder S, Herrero R, Rodriguez A, Bratti MC, Sherman ME, Scarpidis U, Lin QQ, Terai M, Bromley RL, Buetow K, Apple RJ, Burk RD. Human papillomavirus type 16 variants and risk of cervical cancer. J Natl Cancer Inst. 2001;93:315–8.
179. Berumen J, Ordonez RM, Lazcano E, Salmeron J, Galvan SC, Estrada RA, Yunes E, Garcia-Carranca A, Gonzalez-Lira G, Madrigal-de la Campa A. Asian-American variants of human papillomavirus 16 and risk for cervical cancer: a case-control study. J Natl Cancer Inst. 2001;93:1325–30.
180. Refugio Gonzalez-Losa M, Laviada M, Puerto-Solis M, Garcia-Carranca A. Molecular variants of HPV type 16 E6 among Mexican women with LSIL and invasive cancer. J Clin Virol. 2004;29:95–8.
181. Xi LF, Critchlow CW, Wheeler CM, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Hawes SE, Surawicz C, Goldbaum G, Holmes KK, Kiviat NB. Risk of anal carcinoma in situ in relation to human papillomavirus type 16 variants. Cancer Res. 1998;58:3839–44.
182. Xi LF, Koutsky LA, Galloway DA, Kuypers J, Hughes JP, Wheeler CM, Holmes KK, Kiviat NB. Genomic variation of human papillomavirus type 16 and risk for high grade cervical intraepithelial neoplasia. J Natl Cancer Inst. 1997;89:796–802.
183. Matsumoto K, Yoshikawa H, Nakagawa S, Tang X, Yasugi T, Kawana K, Sekiya S, Hirai Y, Kukimoto I, Kanda T, Taketani Y. Enhanced oncogenicity of human papillomavirus type 16 (HPV16) variants in Japanese population. Cancer Lett. 2000;156:159–65.
184. Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J, Kiviat NB. Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. Cancer Epidemiol Biomarkers Prev. 2007;16:4–10.
185. Stoppler MC, Ching K, Stoppler H, Clancy K, Schlegel R, Icenogle J. Natural variants of the human papillomavirus type 16 E6 protein differ in their abilities to alter keratinocyte differentiation and to induce p53 degradation. J Virol. 1996;70:6987–93.
186. Cruz-Hernandez E, Garcia-Carranca A, Mohar-Betancourt A, Duenas-Gonzalez A, Contreras-Paredes A, Perez-Cardenas E, Herrera-Goepfert R, Lizano-Soberon M. Differential splicing of E6 within human papillomavirus type 18 variants and functional consequences. J Gen Virol. 2005;86:2459–68.
187. Chakrabarti O, Veeraraghavalu K, Tergaonkar V, Liu Y, Androphy EJ, Stanley MA, Krishna S. Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras. J Virol. 2004;78:5934–45.
188. Contreras-Paredes A, Cruz-Hernandez E, Martinez-Ramirez I, Duenas-Gonzalez A, Lizano M. E6 variants of human papillomavirus 18 differentially modulate the protein kinase B/phosphatidylinositol 3-kinase (akt/PI3K) signaling pathway. Virology. 2009;383:78–85.
189. Veress G, Szarka K, Dong XP, Gergely L, Pfister H. Functional significance of sequence variation in the E2 gene and the long control region of human papillomavirus type 16. J Gen Virol. 1999;80(Pt 4):1035–43.
190. Kammer C, Tommasino M, Syrjanen S, Delius H, Hebling U, Warthorst U, Pfister H, Zehbe I. Variants of the long control region and the E6 oncogene in European human papillomavirus type 16 isolates: implications for cervical disease. Br J Cancer. 2002;86:269–73.

191. Park JS, Hwang ES, Lee CJ, Kim CJ, Rha JG, Kim SJ, Namkoong SE, Um SJ. Mutational and functional analysis of HPV-16 URR derived from Korean cervical neoplasia. Gynecol Oncol. 1999;74:23–9.
192. May M, Dong XP, Beyer-Finkler E, Stubenrauch F, Fuchs PG, Pfister H. The E6/E7 promoter of extrachromosomal HPV16 DNA in cervical cancers escapes from cellular repression by mutation of target sequences for YY1. EMBO J. 1994;13:1460–6.
193. Rose B, Steger G, Dong XP, Thompson C, Cossart Y, Tattersall M, Pfister H. Point mutations in SP1 motifs in the upstream regulatory region of human papillomavirus type 18 isolates from cervical cancers increase promoter activity. J Gen Virol. 1998;79(Pt 7):1659–63.
194. Hubert WG Variant upstream regulatory region sequences differentially regulate human papillomavirus type 16 DNA replication throughout the viral life cycle. J Virol. 2005;79:5914–22.
195. Du J, Nordfors C, Nasman A, Sobkowiak M, Romanitan M, Dalianis T, Ramqvist T. Human papillomavirus (HPV) 16 E6 variants in tonsillar cancer in comparison to those in cervical cancer in Stockholm, Sweden. PLoS One. 2012;7:e36239.
196. Boscolo-Rizzo P, Da Mosto MC, Fuson R, Frayle-Salamanca H, Trevisan R, Del Mistro A. HPV-16 E6 L83V variant in squamous cell carcinomas of the upper aerodigestive tract. J Cancer Res Clin Oncol. 2009;135:559–66.

Chapter 4
EGFR Inhibitors as Therapeutic Agents in Head and Neck Cancer

Hanqing Liu, Jennifer R. Cracchiolo, Tim N. Beck, Ilya G. Serebriiskii and Erica A. Golemis

Abstract Squamous cell carcinoma of the head and neck (SCCHN) is one of the more challenging cancers to treat. Although great progress has been made over the years, available treatment options are still far from ideal, as epitomized by a 5-year survival rate of only 30–40 %. A unique feature of SCCHN is that elevated expression of the epidermal growth factor receptor (EGFR), a member of the ErbB receptor tyrosine kinase (RTK) family and highly relevant to oncogenic proliferation, occurs in a significant number of cases, which has prompted great interest in utilizing EGFR-targeted therapies to treat this devastating disease. Significant advances in the treatment of SCCHN have been made using EGFR-targeting monoclonal antibodies. Another class of EGFR-targeting inhibitors, tyrosine kinase inhibitors (TKIs), has also shown promise as a potential treatment option. In order to appreciate how these therapeutic agents work and why they fail when they do, it is crucial to explore the biology of the ErbB family members, the signaling pathways that are associated with them, and how they interact with each specific therapeutic agent. This chapter discusses the biology of EGFR and other ErbB family members in SCCHN, and summarizes the current status of the application of EGFR and ErbB inhibitors.

Keywords Epidermal growth factor receptor (EGFR) · Squamous cell carcinoma of the head and neck (SCCHN) · Tyrosine kinase inhibitor (TKI) · Monoclonal antibodies · Erlotinib · Cetuximab · Radiation · Cisplatin · ErbB family

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4.1 Introduction

Approximately 40,000 new cases of squamous cell carcinoma of the head and neck (SCCHN) are diagnosed each year in the USA [1], [2] and over 500,000 cases are diagnosed worldwide, making this the sixth most common cancer in the world [3]. While decades of intensive clinical investigation have yielded some significant treatment advances, the outcome for patients with stage III–IV SCCHN remains dismal, with 5-year survival rates of 30–40 % [4], [5]. Achieving further improvement in the management of locally advanced and regionally metastatic SCCHN represents a significant challenge given the need to preserve complicated tissue structure and function. This review focuses on one important group of tumor-specific therapies, which use targeted inhibition of the epidermal growth factor receptor (EGFR), also known as avian erythroblastic leukemia viral (v-erb-b) oncogene homolog 1 (ErbB1) or human epidermal receptor (HER1), and its family members ErbB2/HER2, ErbB3, and ErbB4, in the management of SCCHN.

Activation of the ErbB family of transmembrane receptor tyrosine kinases (RTKs) is typically associated with growth and development. Four structurally related members compose the ErbB family: ErbB1/EGFR, ErbB2 (HER2-neu), ErbB3 (HER3), and ErbB4. ErbB family members share a common general structure: each of them contains a large extracellular N-terminal region, a single hydrophobic transmembrane-spanning domain, an intracellular juxtamembrane region, a tyrosine kinase domain, and C-terminal region [6]–[9] (Fig. 4.1). ErbB3 differs from the other family members in having a kinase domain that was long thought to be a pseudo-kinase, although, it has now been shown to have weak autophosphorylation capacity [10]. A second variation of the described general structure is observed with ErbB2. ErbB2 does not bind to any known ligands and is principally involved in heterodimerization with other ErbB receptors, to function as a signal transducer [11]–[12]. ErbB proteins were first identified as cancer-relevant in the 1980s when an aberrant form of the human epidermal growth factor (EGF) receptor was found to be encoded by the avian erythroblastosis tumor virus [13]. In human head and neck cancer, EGFR and its family members are oncogenically altered in several ways (Sect. 4.3), presenting both treatment challenges and potentially promising opportunities for successful application of targeted therapies.

Elevated expression of EGFR is found in a significant number of cases of SCCHN [14], [15] and has prognostic value: overexpression of EGFR correlates with resistance to therapy and reduction of overall survival [16]–[18]. As discussed in detail below, EGFR overexpression activates multiple important cancer survival and proliferation signaling effector pathways. Further, EGFR supports the activity of the DNA repair machinery induced by DNA-damaging therapies commonly used in treatment of SCCHN, such as cisplatin or radiation, positioning inhibition of EGFR to enhance the efficacy of these cytotoxic therapies [17]. Although EGFR is by far the most commonly and most richly overexpressed ErbB family member in SCCHN, all other members have also been reported to be overexpressed to some extent (ErbB2, 3–29 %; ErbB3, 21 %; and ErbB4, 26 %; [19]).

Insight into the role of EGFR in SCCHN can be gained from the observation that increased EGFR expression is not only appreciable in SCCHN tumor samples, it has also been observed in “healthy” mucosa samples of patients with SCCHN

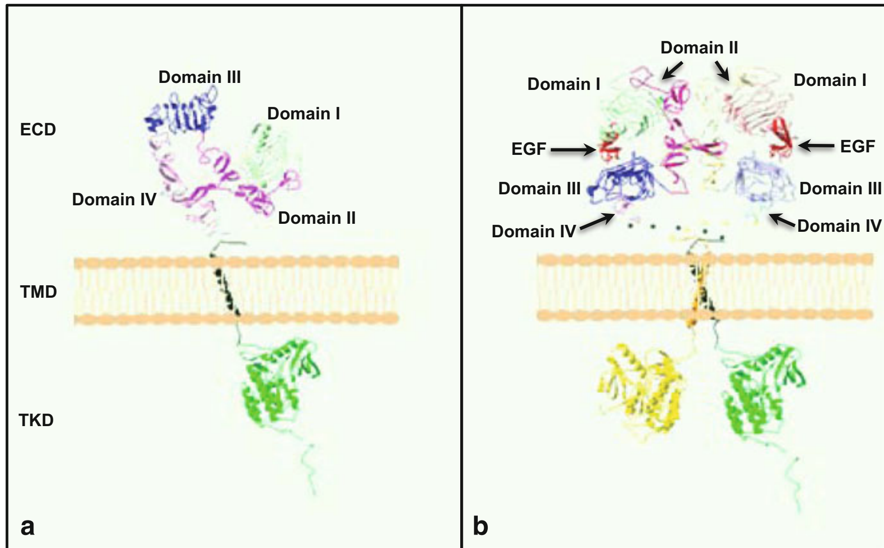

Fig. 4.1 Illustration of ligand-induced dimerization of epidermal growth factor receptor (EGFR). Several PDB files 1nql [8], 1ivo [249], 1m17 [250], 2gs6 [251], and 2jwa [252] were used to propose a complete EGFR crystal structure. **a** Unliganded state (inactive); EGFR exists as a tethered monomer. Domain II interacts with domain IV and domains I and III are far apart. *ECD* extracellular domain, *TMD* transmembrane domain, *TKD* tyrosine kinase domain. **b** Ligand-induced (active), dimeric state of 2:2 EGF-EGFR complex. Domains I and III are closer together and interact with the ligand (EGF in this case) [35]. *EGF* epidermal growth factor

[20]. The work by Grandis et al. provides evidence that tissue adjacent to SCCHN undergoes molecular changes that precede histological changes [14], [15]. The fact that EGFR tends to be overexpressed in noncancerous tissue adjacent to sites of tumor growth makes EGFR a potential biomarker for early stages of malignant transformation in addition to being a therapeutic target [21]. EGFR overexpression even in noncancerous cells may explain why EGFR expression did not correlate with disease stage at presentation, or other known clinical prognostic variables, in stage II–IV carcinomas of the oral cavity, oropharynx and supraglottic larynx, tongue base and hypopharynx in a cohort of 155 patients evaluated by Ang et al. [18]. Human papillomavirus association, a favorable prognostic variable [22], was also not accounted for in this study, which may be an alternative explanation for the lack of observed correlation. However, since the Ang study, it has been suggested that expression of truncated and activated EGFR is associated with advanced tumor and nodal stage [23].

Importantly, Ang et al. did show in the aforementioned 155-patient cohort study that EGFR expression was a strong independent prognostic indicator of 5-year overall survival (40 % for EGFR negative and 20 % for EGFR positive; $p = 0.0006$) as well as disease-free survival (25 % for EGFR negative and 10 % for EGFR positive; $p = 0.0016$). These findings agreed with an earlier study, based on 140 primary laryngeal squamous cell carcinomas, where the 5-year survival rate was 81 % for patients with EGFR negative tumors, compared to only 25 % for patients with EGFR positive tumors. The 5-year relapse-free survival was 77 % for patients

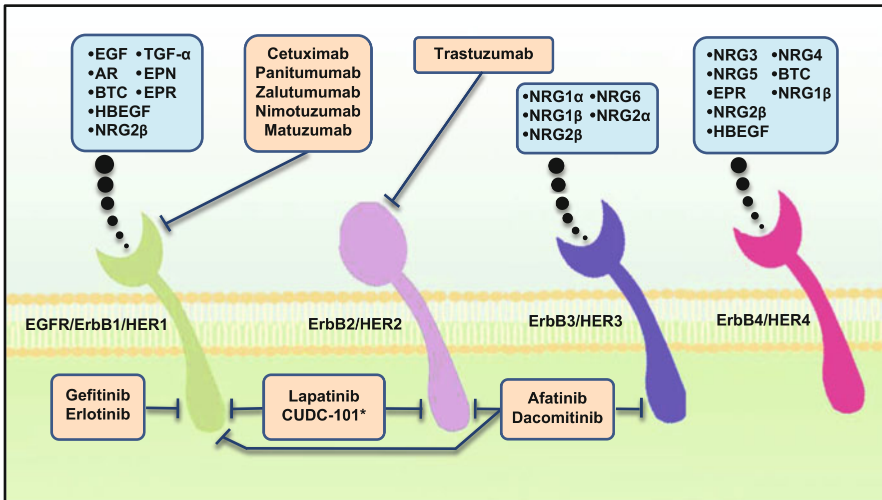

Fig. 4.2 ErbB receptors, ligands, phosphorylation sites, and inhibitors. Ligands are contained within *light blue boxes* and EGFR-inhibitors are contained within *light orange boxes*. Monoclonal antibodies target the extracellular portion of the appropriate receptor and TKIs target the cytoplasmic kinase domain(s) [29], [253]. *CUDC-101 also inhibits histone deacetylase (HDAC) [195]

with EGFR negative tumors compared to 24 % for patients with EGFR positive tumors [24]. Chang et al. have shown that high EGFR expression also correlates with standard treatment (radiotherapy alone) failure in early glottic cancer. In the same study, EGFR expression was also higher in the recurrent group than in the control group [25]. Considering the available evidence, EGFR-targeting therapeutics appear particularly appropriate for the treatment of SCCHN.

Two complementary therapeutic strategies have been developed to target EGFR. The first strategy involves targeting the extracellular domain of the receptor (Fig. 4.2) with monoclonal antibodies, such as cetuximab, panitumumab, and zalutumumab [26], [27] (Sect. 4.4.1; Fig. 4.2; Table 4.1). The second strategy targets the intracellular domain of the receptor with low-molecular-weight tyrosine kinase inhibitors (TKIs, e.g., gefitinib and erlotinib; see Sect. 4.4.2, Fig. 4.2, and Table 4.1) [28]. The nature of EGFR/ErbB signaling as well as therapeutic strategies to manage tumors with EGFR/ErbB involvement are addressed in detail in the remainder of this chapter.

4.2 Regulation of the Activity of EGFR and the ErbB Family In Normal Cells

4.2.1 Ligand Binding and Dimerization: Activation of ErbB Proteins in Normal Cells

The extracellular regions of ErbB family members contain two homologous ligand-binding domains (domain I and III) and two cysteine-rich domains (domains II and IV; Fig. 4.1). The ligands required for dimerization and activation of EGFR, ErbB3,

Table 4.1 EGFR-targeting agents in clinical development [74], [128], [163], [175]

	Target	Stage of development/trials	Administration	Comments
Monoclonal antibodies				
Cetuximab	EGFR; extracellular; domain III	Approved for use in SCCHN/EXTREME	IV, weekly	First targeted therapy for SCCHN
Panitumumab	EGFR; extracellular; domain III	Phase III/PRISM, PARTNER, SPECTRUM (SCCHN)	IV, every 3 weeks	Potentially less immunogenic than chimeric mAbs; low rate of infusion-related hypersensitivity reaction
Zalutumumab	EGFR; extracellular; domain III	Phase III, DAHANCA 19 (SCCHN)	IV, every 2 weeks	Particularly effective induction of antibody-dependent cellular cytotoxicity
Nimotuzumab	EGFR; extracellular; domain III	Phase I/II (SCCHN)	IV, weekly	Binds with less affinity than cetuximab; mild to no skin toxicity
Matuzumab	EGFR; extracellular domain III	No active trials	N/A	Binds at a completely different epitope than cetuximab
Tyrosine kinase inhibitors				
Gefitinib	ATP binding site; intracellular; TK domain of EGFR; reversible binding	Phase III	PO, daily	First TKI to reach a Phase III investigation; trial failures led to its withdrawal from clinical investigation in SCCHN
Erlotinib	ATP binding site; Intracellular; TK domain of EGFR; reversible binding	Phase II and Phase III studies	PO, daily	Investigated as first-line treatment with radiotherapy or chemoradiotherapy in locally advanced SCCHN
Lapatinib (2nd generation)	ATP binding site; intracellular; TK domain of EGFR and ErbB2/HER2; reversible binding	Phase II/III	PO, daily	Dual specificity for EGFR and HER2

Table 4.1 (continued)

	Target	Stage of development/trials	Administration	Comments
Afatinib (2nd generation)	ATP binding site; intracellular; TK domain of EGFR, ErbB2, ErbB4; irreversible binding	Phase III	PO, daily	Multi-specificity; comparable outcome to cetuximab monotherapy in a randomized phase II study
Dacomitinib (2nd generation)	ATP binding site; intracellular; TK domain of EGFR, ErbB2, ErbB4; irreversible binding	Phase II	PO, daily	Activity against wild-type and mutant receptors, including EGFRvIII
CUDC-101	ATP binding site; intracellular; TK domain of EGFR and ErbB2; also histone deacetylase (HDAC)	Phase I	N/A	Structurally contains elements of TKIs (e.g., erlotinib) and elements of HDAC inhibitors (e.g., vorinostat)

and ErbB4 can be separated into five groups: (1) EGFR-specific ligands such as EGF, amphiregulin (AR), epigen (EPN), and transforming growth factor alpha (TGFα); (2) the ErbB3-specific ligands neuregulin1α (NRG1α), NRG2α, and NRG6; (3) NRG3, NRG4, and NRG5 specifically bind ErbB4; (4) the bispecific ligands betacellulin (BTC), epiregulin (EPR), and heparin binding EGF-like growth factor (HBEGF), which bind EGFR and ErbB4, and NRG1β which binds ErbB3 and ErbB4; and (5) NRG2β, which is a pan-ErbB ligand and binds to EGFR, ErbB3, and ErbB4 [29] (Fig. 4.2). ErbB2 does not depend on ligands for dimerization or activation. Instead, domains I and III interact directly in a configuration that renders the ligand-binding site inaccessible [30]. To date, no high-affinity soluble ligand has been identified for ErbB2 [30], [31].

ErbB proteins can homodimerize or heterodimerize [32]. EGFR-EGFR and ErbB4-ErbB4 homodimers and EGFR-ErbB2, EGFR-ErbB3, ErbB2-ErbB3, and ErbB2-ErbB4 heterodimers are abundant in SCCHN tumors and cell lines [19], [33]. The configuration changes associated with dimerization lead to a transient kinase activation in normal cells that becomes constitutive in cancers. The actual activation process involves an asymmetric interaction between intracellular kinase domains that results in auto- or transphosphorylation of ErbB family members [34]–[36]. As ErbB2 is not ligand-responsive, phosphorylation of this kinase can only be activated through heterodimerization, frequently with ErbB3 [12], [36].

4.2.2 ErbB Trafficking and other Mechanisms to Influence EGFR Function in Normal Cells

As with most RTKs, duration of ErbB activation is limited by countervailing regulatory processes. Once ligand bound, internalization removes EGFR from the cell surface. More than one pathway for internalization has been described. In the most studied pathway, binding of the E3 ubiquitin ligase Cbl to phosphorylated Y-1045 of activated EGFR at the plasma membrane triggers clathrin-mediated endocytosis [37]. Multiple additional activation-associated phosphorylations conferred by calmodulin kinase II and p38 enhance the interaction of Cbl with activated EGFR [38], [39]. Subsequently, EGFR is either recycled to the plasma membrane, or alternatively processed through the late endosome and multivesicular body for degradation in the lysosome. An alternative non-clathrin-based endocytosis process has also been described: in this case, the majority of EGFR is targeted for lysosomal destruction [40], [41]. Additional interactions involving the molecular motor dynamin 2 (DYN2) and a scaffolding protein, CIN85, support targeting of EGFR to the lysosome rather than for recycling [42]. As discussed below, reduced phosphorylation of EGFR that limits interaction with Cbl and other internalization proteins often accompanies therapeutic resistance to EGFR inhibitors (EGFRIs).

As Brand et al. have reviewed in detail [43], epithelial cancers such as SCCHN are characterized by a high frequency of nuclear EGFR. At present, it is not clear whether this localization is unique to cancer cells or instead represents an extreme case of

a signaling process that also exists in normal cells. Mechanistically, to enter the nucleus, EGFR is passaged from clathrin-coated pits to the Golgi and subsequently via retrograde transport in COPI vesicles to the endoplasmic reticulum (ER) [44], after which the Sec61 translocon moves EGFR from the inner nuclear membrane to the nucleus [45], [46]. Nuclear EGFR acts as a transcription coactivator for many genes associated with cell proliferation, including BCRP, Aurora-A, cyclin D, Myc, c-Myb, Cox-2, and iNOS, and also binds and supports activity of PCNA and DNA-PK to enhance DNA synthesis and repair [47]. Plausibly, limited exercise of these activities contributes to the pro-proliferative function of EGFR in normal cells.

4.3 Causes and Consequences of Altered EGFR/ErbB Function in SCCHN

4.3.1 Overexpression of EGFR and its Ligands

All members of the ErbB receptor family have been detected in SCCHN at increased expression levels, generally with multiple ErbB receptor family members overexpressed at the same time [48], [49], although conflicting reports regarding ErbB3 and ErbB4 expression levels have been published [48], [50], [51]. EGFR overexpression in SCCHN is often caused by an increase in the number of gene copies [52] and its overexpression correlates with metastasis and poor outcome [16], [18], increased kinase dimerization and activation, and elevated activity of the downstream effector pathways (discussed below). Overexpression of ligands such as TGFα has been linked to a poor prognosis [53], [54] and has been associated with malignant tumor development at a number of different tissue sites in transgenic mice [55]–[57]. Additionally, expression of TGFα [19], AR [58], [59], and HB-EGF [60] (other ligands are likely to also be of importance [61]) has been shown to enhance oncogene-induced carcinogenesis and affect the response of tumor cells to EGFR-inhibition [62]–[65]. Lastly, increased expression of nuclear EGFR has been associated with a higher incidence of local recurrence and inferior disease-free survival in oropharyngeal squamous cell carcinoma [66], [67]. Nuclear EGFR expression levels retained their prognostic significance in multivariate analysis adjusting for well-characterized prognostic variables [66].

4.3.2 Alternative Forms of EGFR Affecting its Activity in SCCHN

Although there are many altered forms of EGFR [68]–[70], Hama et al. only found five different EGFR mutations in 6 out of 82 patients [68]. Another study identified EGFR mutations in only 3 of 127 patients (2.4 %) [69]. A third study found in-frame deletion mutation in exon 19 of EGFR (E746_A750del) in only 3 of 41 larynx, tongue, and tonsil tumor samples [69]. Stransky et al. performed whole-exome sequencing on tumor samples from 92 patients with SCCHN and validated

known relevant mutations in TP53, CDKN2A, PTEN, PIK3CA, and HRAS [71]. Agrawal et al. used the same methods to study 32 primary tumors and six of the genes that were mutated in multiple tumors were reassessed in up to 88 additional SCCHN samples. This study identified mutations in FBXW7 and NOTCH1 in addition to previously identified genes and found that NOTCH1 functioned as a tumor suppressor in head and neck cancer [72]. ErbB family members were not identified as commonly mutated in either study [71], [72]. The one EGFR mutation of note in SCCHN is EGFR variant III (EGFRvIII), which results in a truncation of the ligand-binding domain that results in ligand-independent, constitutive-signaling, greatly potentiating tumorigenicity. EGFRvIII is the most common form of mutant EGFR and has been described in several types of cancer [73]–[78], including SCCHN [68], [79], [80]. However, the reported frequency of EGFRvIII in head and neck cancer is highly inconsistent. The presence of EGFRvIII in SCCHN ranged from none [68] to 15 % [81] to 42 % [80] and may vary by specific SCCHN subsite [82]. Sok et al. reported that EGFRvIII-transfected SCCHN cells showed increased proliferation in vitro and increased tumor volumes in vivo compared with vector-transfected cells. Furthermore, EGFRvIII-transfected SCCHN cells showed decreased apoptosis in response to cisplatin and decreased growth inhibition following treatment with cetuximab compared with vector-transfected control cells. It was not established if the transfected cells expressed EGFRvIII at levels similar to those observed in actual patient samples [80].

4.3.3 Consequences of EGF/ErbB Activation

Dimerization of the ErbB RTKs can result in the constitutive activation of five different major intracellular signaling pathways, each of which contributes to the oncogenic activity of this kinase family in SCCHN. These pathways are the Ras/Raf/MEK/ERK kinase pathway [83], the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [84], the signal transducer and activator of transcription (STAT) pathway [85], the phospholipase C (PLC) and protein kinase C (PKC) [86], [87] pathway, and the Src kinase pathway [88] (Fig. 4.3).

4.3.3.1 Ras/Raf/MAPK

Increased activity of the Ras/Raf/MAPK pathway initiated by EGFR signaling is strongly linked to tumorigenesis in SCCHN [83]. Following EGFR autophosphorylation, mainly on residue Y1068 and Y1086, the growth factor receptor-bound protein 2 (GRB2) adaptor protein is either directly recruited through binding of its Src Homology 2 (SH2) domain to the phosphotyrosine residues of the activated receptor, or, alternatively, GRB2 is indirectly recruited to active EGFR by interaction with the Src homolog and collagen homolog (SHC) adaptor protein, which directly binds tyrosine-phosphorylated sites on EGFR, itself is tyrosine phosphorylated, and then binds GRB2 [89]. EGFR-bound GRB2 subsequently recruits and activates guanine nucleotide exchange factor Son of Sevenless (SOS). Activated SOS

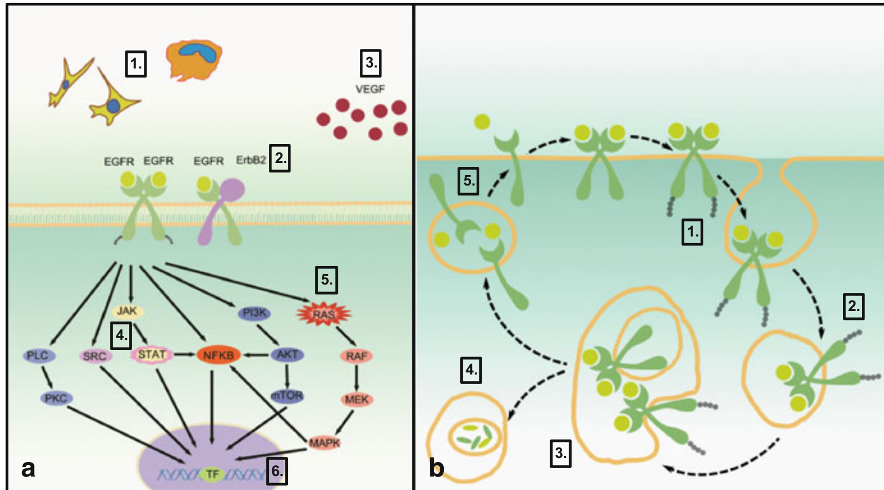

Fig. 4.3 Major downstream pathways of EGFR and proposed mechanisms of resistance to EGFR-targeted therapy. **a Six major signaling pathways** downstream of EGFR have been linked to tumorigenesis of SCCHN and/or resistance to EGFR-inhibitors. The first pathway involves PLC and PKC, where phosphorylated EGFR recruits PLC and subsequently activates PKC to affect cell cycle regulation, cellular survival, malignant transformation, and apoptosis [98], [99]. The second pathway involves Src kinases, which play a fundamental role in many cellular events, including the regulation of cell proliferation, migration, adhesion, and tumor angiogenesis [103]–[105]. The third pathway involves JAK/STAT and is implicated in the activation of genes associated with tumorigenesis and cell survival [93]. The fourth pathway involves NF-κB and has been directly linked to tumorigenesis, metastasis, and chemoresistance in SCCHN [110]–[114]. The fifth pathway involves PI3K/Akt/mTOR, which play a cardinal role in cancer cell metabolism, cell growth, proliferation, angiogenesis, metabolism, differentiation, motility, and survival [92]. The sixth pathway involves Ras/Raf/MAPK; phosphorylated EGFR activates Ras and subsequently stimulates Raf and the MAPK pathway to affect muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, cell proliferation, tumor invasion, and metastasis [83]. **Proposed mechanisms of EGFR-targeted therapy resistance**: *1* the microenvironment, including cancer associated fibroblasts (CAFs), plays a role in the resistance to EGFR-targeted therapy; *2* ErbB2 amplification has been demonstrated to be involved in *de novo* and acquired resistance to EGFR targeted therapy; *3* VEGF expression has been shown to be a prognostic marker in SCCHN; *4* STAT3 expression has been implicated in the resistance to EGFRIs in head and neck tumors; *5* KRAS mutations have been detected in SCCHN patients; *6* emerging evidence has shown connections between epigenetic changes and development of resistance to multiple cancer therapeutics. **b Ubiquitination and trafficking of EGFR** has been implicated in resistance to EGFR-inhibitors: *1* ubiquitination, *2* early endosome, *3* late endosome, *4* lysosome, *5* recycling endosome

increases the pool of active, GTP-bound Ras, inducing a kinase cascade involving c-Raf, MEK1/2, and ERK1/2. Phosphorylated ERK1/2 translocates into the nucleus and activates transcription factors that induce transcription of many genes promoting cell growth and survival; a residual pool of active cytoplasmic ERK1/2 also phosphorylates cytoskeletal proteins such as actin, which promotes cell motility, and regulators of cell division and cytokinesis, vesicle and organelle movement, and mitochondrial targets such as Bcl2 that render cells resistant to apoptosis (Fig. 4.3) [89], [90].

4.3.3.2 PI3K/Akt/mTOR

Dimerization of EGFR or ErbB2 with ErbB3 is strongly associated with PI3K activation, because of the high prevalence of PI3K-activating docking sites on ErbB3 [91]. PI3Ks are composed of a catalytic p110 and a regulatory p85 subunit. The p110 subunits catalyze the phosphorylation of phosphatidylinositol 4,5-diphosphate (PIP2) to the second-messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn phosphorylates and activates the protein serine/threonine kinase Akt (also known as protein kinase B), inducing protein synthesis and cell growth through activation of the mTOR effector pathway, and limiting the apoptotic machinery (Fig. 4.3) [92]. Detailed discussion of the PI3K/Akt/mTOR pathway in SCCHN can be found in Chap. 6 of this book.

4.3.3.3 STAT

The signal transducers and activators of transcription (STAT) proteins were originally identified as downstream effectors of non-tyrosine kinase cytokine receptors, such as IL-6, IL-22, IFN-α/β, and IFN-λ. However, STATs can also be directly activated by EGFR, or by EGFR effectors such as c-Src [85], and constitutive activation of STATs has been reported in SCCHN [93]. Activated STATs migrate from cytoplasm to nucleus and upregulate the expression of many proteins associated with tumorigenesis, including the prosurvival factor NF-κB [94]. Detailed characterization of the JAK/STAT pathway is found in Chap. 7 of this book.

4.3.3.4 PLC/PKC

PLC is recruited by phosphorylated EGFR and subsequently activated. Once activated, PLC hydrolyzes PIP2 to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG in turn activates members of the PKC family (which is composed of 12 different isoforms in mammals; Fig. 4.3) [95]. It has been shown that primary tumors express elevated levels of total and phosphorylated PLCγ (one of six isotypes: β, γ, δ, ε, ζ, and η; [96]) and that EGFR-stimulated activation of PLCγ promotes invasion of SCCHN [97]. PLCγ inhibition decreases the invasive potential of prostate, breast, and head and neck carcinoma cells [98], [99]. Protein kinase Cε [100] is a promising prognostic factor for relapse and overall survival of SCCHN [101]. PKCζ is highly expressed in SCCHN tumors and mediates EGF–induced growth of SCCHN tumor cells by regulating MAPK [87].

4.3.3.5 Src

Activation of members of the Src kinase family (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes, and Yrk; [102]) by EGFR and ErbB2 also positively regulates cell proliferation, migration, adhesion, and tumor angiogenesis, with activation seen in

many cancer types, including SCCHN [103]–[105]. In SCCHN, Src contributions to EGFR-dependent activation of STAT3 and STAT5 are important for tumor growth [106]. Reciprocally, Src helps to activate EGFR by participating in G protein-coupled receptor-initiated TGFα release [107]. Changes in the interaction between Src and EGFR have been suggested to be involved in resistance to cetuximab by increasing translocation of EGFR to the nucleus (Sect. 4.2.2; Fig. 4.3) [105], [108]. Src additionally interacts with other RTKs that are upregulated during acquisition of resistance to EGFRIs, such as IGF-1R (insulin-like growth factor-1 receptor) and others [109].

4.3.3.6 Nuclear Factor-κB (NF-κB)

High expression and constitutive activation of the transcription factor nuclear factor-κB (NF-κB) has been directly linked to tumorigenesis, metastasis, and chemoresistance in many cancers including SCCHN [110]–[114], with particularly high levels of NF-κB in highly metastatic cells [110], [115]. NF-κB induction of matrix metalloproteinase-1, -2, -9, and -14 fibronectin and ß1 integrin, and vascular endothelial growth factor C, is strongly associated with tumor progression and metastasis [115]. In SCCHN, NF-κB activation has been described as both independent of and dependent on EGFR signaling [116]–[118]. In the EGFR-dependent activation of NF-κB, phosphorylated EGFR activates PI3K, ERK1/2, and STAT3, all of which are associated with increased NF-κB activity (Fig. 4.3) [116]. RNAi knockdown of components of the NF-κB pathway and pharmacologic inhibition of NF-κB enhanced cell death induced by erlotinib in EGFR-mutant lung cancer cells [119] support relevance of this signaling axis.

4.4 EGFR and Targeted Inhibitors

Organ-preservation strategies in the treatment of SCCHN generally entail DNA-damaging radiation and chemotherapies. For patients with locally advanced disease, randomized control trials have shown that the addition of chemotherapy to radiotherapy improves both locoregional control (62 % for chemoradiotherapy compared to 42 % for radiation alone; [120]) and overall survival (23 % for radiation alone and 37 % for chemoradiotherapy; [121]) compared with radiation therapy alone [120], [121]. Although chemoradiation has resulted in an improvement in local control and survival, managing the significant associated toxicity is a formidable challenge [122].

Targeting EGFR has emerged as an avenue to further improve treatment of SCCHN. EGFR inhibition seems to prevent activation of DNA repair mechanisms that enable cancer cells to survive radiation- or chemotherapy-induced DNA damage [123], [124]. However, it is important to note that a 10 % cetuximab monotherapy overall response rate in patients with platinum-refractory SCCHN has been reported [125], which suggests a secondary therapeutically relevant mechanism independent

of sensitization to DNA damaging agents. It is unknown if this observation is due to cell-mediated immunity or if it is a reflection of the proportion of cancers that are truly EGFR dependent. Numerous data suggest that EGFR may accelerate repopulation, a condition of enhanced cellular proliferation after exposure to ionizing radiation, contributing to the radioresistance associated with head and neck cancers [126], [127]. Despite potential secondary mechanisms, preclinical and clinical data support the premise that the inhibition of EGFR activity increases radio- and chemosensitivity of SSCHN tumors [126]–[128]. These findings provide support that EGFRIs act as radiation- and chemotherapy-"sensitizers," making them ideal adjuncts to current treatment paradigms for SCCHN. However, conflicting data regarding the sensitizing potential of EGFRIs do exist: early data from the RTOG 0522 phase III trial suggest that the addition of the EGFRI cetuximab (Sect. 4.4.1.1) does not improve response rates to chemoradiation in patients with stage III and IV SCCHN. It was proposed that exposure to cetuximab was suboptimal in the study and that patient selection may have impacted the results, considering that the study reported among the highest 2-year survival rates for both the control and the experimental group [129].

Several agents that target EGFR are now established or under active investigation and have shown great promise (Table 4.1). Also noteworthy is that the side effect profiles of EGFRIs have been generally favorable compared to standard chemotherapeutics [130]–[133]. Monoclonal antibodies and small molecule TKIs are the two major classes of anti-EGFR agents.

4.4.1 Monoclonal Antibodies

Monoclonal antibodies targeting ErbB receptors play a significant role in the treatment of SCCHN. Cetuximab, the pioneer for antibody-based anti-ErbB therapy in SCCHN, was approved for treatment of locally or regionally advanced SCCHN in 2006 [128] and for metastatic SCCHN in 2011 [134]. While cetuximab represents the EGFR inhibitor with the most clinical data and the most significant results in the treatment of SCCHN, multiple additional antibodies targeting ErbB receptors are currently being investigated in clinical trials (Table 4.1). The successes of cetuximab and other receptor-targeting antibodies were not achieved overnight. Promising results of EGFR-targeting antibodies used in mice were first published in 1984 [135], over two decades before cetuximab was approved for clinical use. This section introduces several relevant antibodies and covers their current status as it pertains to SCCHN.

4.4.1.1 Cetuximab

Cetuximab is a chimeric monoclonal antibody that inhibits EGFR by binding to its extracellular domain (Fig. 4.2). Cetuximab binds to EGFR with a higher affinity than its natural ligands EGF and TGFα [136]–[138]. Once bound to EGFR domain

III, cetuximab occludes the ligand-binding site, thus inhibiting ligand-dependent EGFR signaling [139]. Depletion of the targeted receptors from the cell surface via downregulation is a second mechanism of effective EGFR inhibition [140]. Additionally, binding of cetuximab to EGFR enhances antibody-dependent, cell-mediated cytotoxicity via natural killer cells and macrophages [141], [142].

Cetuximab has been approved for the treatment of three subpopulations of patients with SCCHN. These subpopulations include: patients with locally or regionally advanced SCCHN (cetuximab in combination with radiation therapy; [128]); patients with recurrent or metastatic platinum-refractory SCCHN (cetuximab monotherapy; [143]); and patients with recurrent locoregional and/or metastatic SCCHN not refractory to platinum-based therapies (cetuximab in combination with platinum chemotherapy and 5-fluorouracil as first-line therapy; [128]).

Phase I studies of cetuximab defined the dose and schedule required to maintain biologically active and tolerable levels [144], [145]. Whether used in combination with chemotherapy or radiotherapy, or as monotherapy, cetuximab was found to have nonlinear saturation kinetics. Median serum cetuximab terminal half-life ranged from 14 to 97 h with doses from 5 to 300 mg/m^2. Skin reactions increased significantly at doses of 500 mg/m^2 or higher. Given the results of these phase I trials, the recommended cetuximab regimen was established as an initial loading dose of 400 mg/m^2 followed by weekly doses of 250 mg/m^2 [144], [145].

The landmark cetuximab phase III study published by Bonner and colleagues involved 424 patients and led to the approval of cetuximab for the treatment of patients with locally or regionally advanced SCCHN (cetuximab in combination with radiation therapy) [128]. This study compared cetuximab and high-dose radiotherapy with high-dose radiotherapy alone. The findings showed that cetuximab and radiotherapy significantly improved median overall survival and median progression-free survival when compared to radiation alone [128]. The 5-year follow-up of the Bonner study showed an absolute survival increase of 9 % in the treatment group receiving cetuximab in combination with radiation therapy (45.6 % cetuximab/radiation vs. 36.4 % radiation alone) [146].

In the case of patients with platinum-refractory recurrent or metastatic SCCHN, Trigo et al. observed an overall response rate to cetuximab monotherapy of 16.5 % and a median survival of 175 days in a single-arm study of 103 patients [143]. The observed response and survival rate were similar to rates expected with platinum therapy alone in chemotherapy naïve patients, prompting interest in exploring combination therapy of cetuximab and chemotherapeutics [147].

In a phase III randomized trial, Burtness et al. compared the impact of cisplatin plus a placebo with the impact of cisplatin plus cetuximab in a population of patients with recurrent and metastatic SCCHN. Burtness et al. demonstrated that the addition of cetuximab resulted in a significant increase in the objective response rate (26 % response rate for cisplatin/cetuximab and 10 % response rate for cisplatin/placebo; $p = 0.03$). Progression free survival (PFS) was not significantly improved, presumably because the control group had better PFS than expected and the sample size was not adequate to observe a significant increase in PFS. Unexpectedly, Burtness et al.

also observed that the activity of cetuximab was not clearest in patients with the highest EGFR staining density and intensity. It was hypothesized that several factors may have contributed to this observation: the small sample size ($n = 123$); suboptimal cetuximab dosing for cases of high density EGFR occurrence; stoichastic interactions at high EGFR density; or constitutive downstream signaling not accounted for in the study [148].

Vermorken et al. conducted a phase III clinical trial (EXTREME trial), similar to the Burtness study, to investigate the efficacy and safety of platinum, fluorouracil (5-FU), and cetuximab as first-line treatment of recurrent and metastatic SCCHN in 442 patients. The EXTREME phase III trial randomly assigned patients to receive cisplatin or carboplatin plus 5-FU and cetuximab or platinum plus 5-FU alone. Six cycles of chemotherapy were the limit for both arms of the study. The addition of cetuximab to chemotherapy significantly increased median overall survival (10.1 months in the cetuximab group and 7.4 months in the chemotherapy-alone group; $p = 0.04$) and progression-free survival (5.6 months in the cetuximab group to 3.3 months in the chemotherapy-alone group; $p < 0.001$) when compared to standard chemotherapy alone [128]. Importantly, additional analysis of the EXTREME data provided further evidence that, in the case of SCCHN, the EGFR expression level is not a clinically useful predictive biomarker [149].

4.4.1.2 Additional EGFR-Targeting Antibodies

Panitumumab is a fully humanized immunoglobulin IgG2 monoclonal antibody that, like cetuximab, binds to EGFR domain III and, in the process, inhibits EGF and TGFα binding [139]. In contrast to cetuximab, panitumumab does not mediate antibody-dependent cellular cytotoxicity and has been shown to have a very low rate of infusion-related hypersensitivity reaction [150]. A phase I study of panitumumab, carboplatin, paclitaxel, and radiation for locally advanced disease has indicated that this combination is feasible with tolerable toxicity. In the phase I study, Wirth et al. showed 69 % of patients had a complete response and 34 % had a partial response [151]. A second trial, SPECTRUM (phase III; NCT00460265), compared cisplatin/5-FU plus panitumumab to cisplatin/5-FU alone in patients with metastatic/recurrent SCCHN. The addition of panitumumab to chemotherapy did not significantly improve median overall survival versus chemotherapy alone but did improve median PFS (5.8 vs. 4.6 months) [138].

Zalutumumab is a human IgG1 high affinity antibody also targeting EGFR domain III and, just like panitumumab and cetuximab, is thought to block ligand binding, but with exceptional tumor specificity at lower doses [139]. In 286 patients with metastatic/recurrent SCCHN after failure of platinum-based therapy, zalutumumab plus best supportive care was compared with best supportive care plus methotrexate at the investigator's discretion [152]. Zalutumumab did not increase overall survival, although progression-free survival was extended [138]. A phase III trial is currently underway (DAHANCA 19; NCT00496652) to determine if the addition of zalutumumab to radiotherapy improves locoregional control.

Nimotuzumab has been approved for SCCHN in several countries, not including the USA. Nimotuzumab is a humanized murine IgG1 monoclonal antibody that also blocks interaction between ligand and receptor by binding to EGFR domain III, but with lesser affinity than some of the other antibodies [153]. The therapeutic implications of this reduced affinity are unclear, but nimotuzumab has been shown to have mild to absent skin toxicity, eliminating a significant toxic effect commonly associated with cetuximab [154]. An early pharmacodynamic study showed nimotuzumab plus radiotherapy was tolerated with no evidence of skin rash in patients with unresectable SCCHN [155]. In a double-blind trial, patients with unresectable locoregional SCCHN were assigned randomly to receive first-line therapy with nimotuzumab plus radiotherapy versus placebo plus radiotherapy [156]. Complete response rates were significantly better in the nimotuzumab group with 59.5 % for patients receiving nimotuzumab and radiotherapy versus 34.2 % of patients receiving radiotherapy alone. Hence, nimotuzumab may offer an EGFR-targeted therapy with a more favorable side effect profile. Further studies are ongoing.

Matuzumab, another humanized mouse monoclonal antibody, also binds to EGFR domain III, but at a completely different epitope than the previously mentioned antibodies. This was confirmed by experiments in which cetuximab and matuzumab were observed to simultaneously bind to EGFR [157]. When bound to EGFR, matuzumab was determined to predominantly prevent domain II from assuming the configuration, in relation to domain III, necessary for high-affinity ligand binding [139], interrupting EGFR signaling. Matuzumab has been evaluated in a phase I dose escalation study focused on patients with advanced EGFR-positive oesophagogastric cancer. At 800 mg/week, matuzumab in combination with epirubicin, cisplatin, and capecitabine (ECX) was well tolerated. Furthermore, in skin biopsies, decreased phosphorylation of EGFR and MAPK was detected [158]. Surprisingly, the phase II study of matuzumab in combination with ECX did not increase response or survival in patients with metastatic oesophagogastric cancer [159]. Matuzumab has not been tested in SCCHN.

4.4.1.3 Monoclonal Antibodies Targeting Other ErbB Proteins

Given the heterodimerization of EGFR with other ErbB family proteins, and the fact that overexpression of some of these proteins can compensate for EGFR inhibition during development of therapeutic resistance, exploring inhibition of additional ErbB family members in SCCHN was a natural development [160], [161]. The ErbB2/HER2-targeting antibody trastuzumab is an invaluable drug for breast cancer and other epithelial tumors [162]–[165]. In vitro studies have shown that trastuzumab enhances the efficacy of gefitinib [166] and cetuximab [167] in SCCHN cells. Surprisingly, analysis of the mRNA expression of EGFR and ErbB2 indicated lack of correlation with efficacy of the combination therapy [167]. Moreover, an independent study found that a subset of non-ErbB2-amplified SCCHN cells was nevertheless extremely responsive to the small molecule multi-ErbB inhibitor lapatinib, based on

activation of a neuregulin-ErbB3 loop [168]. Efforts are underway, although not yet in SCCHN, to develop and evaluate ErbB3- and ErbB4-targeting antibodies. Pertuzumab [169], which binds the ErbB2 dimerization domain, blocks its interaction with all four ErbB family members. MM-121, targeting ErbB3, has been shown to be active in cancers characterized by ligand-induced ErbB3 signaling [170], [171]. Several groups have developed bispecific single-chain antibodies that simultaneously target ErbB2 and ErbB3 (see e.g., [172], [173]). Monoclonal antibodies targeting ErbB4 are also in development [174], [175]. All of these are likely to be promising for development as therapies for SCCHN.

4.4.2 Tyrosine Kinase Inhibitors (TKIs) Targeting EGFR

TKIs block EGFR activation by inhibiting the cytoplasmic tyrosine kinase domain and have proven valuable agents in a number of cancer types, such as non-small cell lung cancer (NSCLC). First-generation TKIs, including gefitinib and erlotinib, reversibly bind the ATP-binding pocket of the kinase domain and are EGFR specific. Second-generation TKIs relevant to SCCHN, including lapatinib, afatinib, and dacomitinib, target multiple ErbB members and in the case of afatinib and dacomitinib irreversibly so (Table 4.1; Fig. 4.2) [136], [176].

Gefitinib, an orally administered, small-molecule, reversible EGFR TKI, was the first TKI to reach phase III trials; however, overall results have dampened expectations. Early studies suggested a clinical benefit of gefitinib similar to cetuximab [177], [178]; unfortunately, more recent results do not indicate significant improvements through the addition of gefitinib to standard therapies [176], [179], [180]. In treatment-refractory SCCHN, gefitinib when compared to methotrexate did not improve overall survival [181]. These findings were consistent when gefitinib was administered orally at 250 or 500 mg daily, despite the fact that earlier studies had indicated favorable response rates and survival with treatment regimens of 500 mg/day [177], [181]. It was originally established that gefitinib produces adverse events that are still manageable and reversible up to 600 mg/day, with dose-limiting toxicity observed at 1,000 mg/day [182]. In a phase II study of 44 patients with SCCHN, Perez et al. investigated if doses higher than 500 mg/day would produce increased skin toxicity (Sect. 4.4.3), which is thought to be associated with improved response to EGFRIs. Patients treated with 750 mg/day did indeed have increased incidence of skin toxicity compared with patients receiving 500 mg/day (58 and 9 % grade 2 skin toxicity for 750 and 500 mg/day, respectively); however, the higher dose of gefitinib failed to significantly improve outcome [183]. Gefitinib (250 mg/day) in combination with docetaxel (docetaxel/gefitinib vs. docetaxel alone) was recently evaluated in a phase III trial of patients with metastatic or locally recurrent SCCHN. In this study, Argiris et al. were unable to demonstrate a statistically significant survival benefit for patients receiving the docetaxel/gefitinib combination. However, subgroup analysis revealed that for patients younger than 65 years of age, the addition of gefitinib to

docetaxel did increase survival significantly (median survival of 7.6 vs. 5.2 months; $p = 0.04$) [184].

Erlotinib, the second first-generation TKI, remains under clinical development and has yielded some encouraging results. Like gefitinib, erlotinib is an orally administered, small molecule, reversible TKI. In 115 patients with refractory SCCHN, erlotinib was superior when compared to palliative chemotherapy [185]. Disease stabilization was maintained in 38.3 % of patients for a median duration of 16.1 weeks. The median progression-free survival was 9.6 weeks and the median overall survival was 6.0 months. Further favorable results were seen in metastatic or recurrent SCCHN patients. In these phase I/II trials, the addition of erlotinib to cisplatin found an overall response rate of 21 %, progression-free survival of 3.3 months, and overall survival 7.9 months [186]. Erlotinib is currently being evaluated in multiple phase II and phase III studies including studies focused on erlotinib combined with cetuximab, carboplatin, and paclitaxel (NCT01316757), docetaxel and radiation therapy (NCT00720304), docetaxel and cisplatin or carboplatin (NCT01064479), and cisplatin (NCT00410826 [176]).

Lapatinib has dual specificity, targeting EGFR and ErbB2 [187]. Surprisingly, in a phase II study focused on recurrent/metastatic SCCHN, lapatinib, as monotherapy, failed to induce any objective responses. And although ErbB2 levels were significantly decreased, EGFR-phosphorylation levels remained unaffected [188]. Lapatinib may still prove to work well in other settings. Ongoing clinical trials are exploring this dual-action small molecule drug as an adjuvant to postoperative chemoradiation in SCCHN patients (NCT00424255) as well as in combination with capecitabine in patients with metastatic SCCHN (NCT00424255).

Afatinib, a second multi-specific TKI, irreversibly targets three of the four ErbB family members: EGFR, ErbB2, and ErbB4 [189]. In a comparison to cetuximab, afatinib showed similar antitumor activity in patients with recurrent or metastatic SCCHN after failing platinum therapy. Median progression-free survival was 15.9 weeks with afatinib and 15.1 weeks with cetuximab. Afatinib is currently being further evaluated in several clinical studies [190]. Patients treated for SCCHN, who are without evidence of disease after post-operative radiochemotherapy, are randomized to afatinib or placebo for 18 months in a phase III trial designed to detect an improvement in disease-free survival (NCT01427478).

Dacomitinib, like afatinib, is an irreversible inhibitor of EGFR, ErB2, and ErbB4 [191]. Dacomitinib is a potent inhibitor of wild-type EGFR as well as EGFR with activating mutations. Furthermore, dacomitinib appears to be active against the T790M secondary EGFR mutation, which generally renders cancer cells resistant to erlotinib and gefitinib, in NSCLC [192]. Razak et al. reported that in a phase II study of patients with recurrent/metastatic SCCHN, dacomitinib had objective response rates (13 %) similar to cetuximab, without infusion-related side effects. Compared to phase II studies involving gefitinib or erlotinib, dacomitinib produces favorable outcomes in terms of disease control and survival [193]. Further phase I clinical trials

investigating the efficacy of dacomitinib in treating SCCHN are under investigation (NCT01737008) [190].

CUDC-101, the small molecule inhibitor 7-(4-(3-Ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-*N*-hydroxyheptanamide, is a novel, multitargeted hybrid anticancer drug candidate. CUDC101 effectively inhibits EGFR, HER2, and histone deacetylase (HDAC) and has shown impressive activity in in vitro and in vivo cancer models [194], [195]. This hybrid inhibitor is currently being investigated in combination with cisplatin and radiation therapy in patients with locally advanced head and neck cancer as part of a phase I drug escalation trial (NCT01384799).

4.4.3 Toxicity and Tolerance

The reported toxicity profiles of chemotherapeutic regimens including EGFRIs are somewhat inconsistent, with some reports suggesting minimal additional toxicity or impact on quality of life [128], [196], whereas others have reported that the addition of EGFRIs resulted in higher rates of toxicity leading to lower treatment compliance [197], [198]. Therefore, despite the fact that the Bonner study did not find significant differences in the rate, intensity, or duration of mucositis, xerostomia, pain, or dysphagia in patients receiving radiation and cetuximab compared with patients receiving radiation alone [128], considering the disputing evidence, it is advisable to use EGFRIs with caution, to be prepared for common adverse events, and to treat the adverse events as recommended [198]. Allergic and anaphylactoid reactions can be observed in patients treated with cetuximab and, less often, with panitumumab administration; however, both of these monoclonal antibodies have fewer nonspecific and hematopoetic side effects compared to other chemotherapeutics [199], [200]. Electrolyte abnormities, specifically hypomagnesemia, are also commonly observed and should be monitored during treatment with EGFRIs [148].

Given that EGFR is expressed at the basal layer of the epidermis, it is not surprising that skin toxicity defines the side-effect profile for EGFRIs (monoclonal antibodies as well as TKIs) [201], [202]. Acneiform eruptions, usually a sterile folliculitis, are the earliest and most characteristic side effect of EGFR inhibition. The rash seems to be dose dependent and is seen in over 50 % of treated patients [201]. The eruption is most often confined to the face, neck, retroauricular area, shoulders, and the upper trunk. Data support the idea that skin toxicity can be a predictive marker of response in colorectal cancers [203]. In a phase II clinical trial the greatest benefit in survival of cetuximab-treated patients was seen in those with a grade 3 rash [204]. Further investigations are necessary to explore the exact mechanism of anti-EGFR-therapy-triggered incidence of rash, how this relates to treatment outcome, and how to best manage this side effect to maximize the quality of life of the patients [198], [200].

4.5 Mechanisms of Resistance to anti-EGFR Therapies

Patients initially responsive to anti-EGFR therapy often develop resistance during the course of treatment [95]. A number of specific factors associated with resistance have been identified. These include altered ubiquitination and trafficking (Sect. 4.5.1) [205]–[207]; overexpression and amplification of ErbB2 (Sect. 4.5.2) [208]; altered expression levels of VEGF (Sect. 4.5.3) [209]; altered expression levels of STAT3 (Sect. 4.5.4) [210]; KRAS mutations (Sect. 4.5.5) [211], [212]; changes in the tumor microenvironment (Sect. 4.5.6) [213]; epigenetic compensation (Sect. 4.5.7) [214]; and several other factors (Sect. 4.5.8 and Fig. 4.3).

4.5.1 Altered Ubiquitination and Trafficking of EGFR

Altered EGFR ubiquitination represents a mechanism of acquired resistance to cetuximab [205]–[207]. In vitro resistance to cetuximab was established by exposing cells to subeffective doses of cetuximab [207] or by prolonged exposure to escalating doses [206]. Lu et al. found that the cetuximab-resistant colorectal cancer cell line DiFi5 (rendered resistance through prolonged exposure to cetuximab) had markedly lower levels of EGFR. However, DiFi5 cells had enhanced associations between EGFR and the E3 ubiquitin ligase Cbl, as well as increased levels of ubiquitinated EGFR. DiFi5 also had significantly higher levels of active, Y16-phosphorylated Src, both at baseline and post-EGF stimulation, with inhibition of Src with the nonselective kinase inhibitor PP2 reversing cetuximab resistance. In addition, DiFi5 cells responded to EGF stimulation with more robust phosphorylation of EGFR at Y845 and strong phosphorylation of Akt and other extracellular EGFR signal–regulated kinases. These observations suggest that colorectal cancer cells may develop resistance to cetuximab by reducing EGFR levels via increased ubiquitination and degradation and via increased Src kinase-mediated cell signaling to bypass dependency on EGFR for cell growth and survival [207].

On the other hand, Wheeler et al. reported increased EGFR expression levels associated with deregulation of EGFR internalization and degradation in several resistant clones of NSCLC cell lines [205]. Loss of c-Cbl association with EGFR was reported to significantly lessen EGFR ubiquitination after EGF stimulation in the cetuximab-resistant cells compared to the nonresistant parent cells. These findings suggest that acquired resistance to cetuximab is accompanied by deregulation of EGFR internalization/degradation and subsequent EGFR-dependent activation of ErbB3 [205]. Further supporting the role of decreased EGFR ubiquitination in treatment resistance, Ahsan et al. found that cisplatin-resistant head and neck cancer cell lines undergo minimal EGFR phosphorylation at the Y1045 site and minimal ubiquitination [215].

4.5.2 ErbB2 Amplification

Ritter et al. demonstrated elevated levels of phosphorylated EGFR, EGFR/ErbB2 heterodimers, TGFα, hairpin-binding EGF, and heregulin RNA in trastuzumab-resistant human breast cancer cells. These findings suggest that enhanced EGFR-mediated activation of ErbB2 may be a potential mechanism of acquired resistance to trastuzumab [216]. Another recent study by Yonesaka et al., identified a new mechanism of *de novo* and acquired resistance to cetuximab via increased signaling through ErbB2. Yonesaka et al. have shown that amplification of ErbB2 or upregulation of heregulin (ErbB3/ErbB4 ligand) is present in cetuximab-resistant colorectal cancer patients. This study suggests that ErbB2 inhibitors, in combination with cetuximab, represent a rational therapeutic strategy that should be assessed in patients with cetuximab-resistant SCCHN [161].

Erjala et al. observed that increased expression levels of phosphorylated ErbB2 and total ErbB3 were associated with SCCHN cell line resistance to gefitinib [208]. Confirming the importance of ErbB2 in resistance: when gefitinib was combined with pertuzumab, an antibody targeting ErbB heterodimerization, significant growth inhibition of relatively gefitinib-resistant SCCHN cell lines was observed. Phosphorylated ErbB2 and total ErbB3 were not predictive of resistance to cetuximab [208].

4.5.3 VEGF Expression

Enhanced angiogenesis is a fundamental step in the transition of tumors from a dormant state to a malignant one and correlates with tumor progression and metastasis [217]. Angiogenesis is elevated in various human tumors, including SCCHN, and VEGF has been demonstrated to be a major angiogenic factor [218]. Preclinical and early clinical data imply a central role of angiogenesis in SCCHN: up to 90 % of SCCHNs express vascular endothelial growth factor (VEGF) and the respective receptors (VEGFRs) [219]. Multiple studies support the prognostic implications of angiogenic markers in SCCHN [220]. It has been shown that EGFR activation and the overexpression of three major ErbB-associated ligands trigger upregulation of multiple VEGF members and may induce resistance to anti-EGFR agents in vitro [209].

Riedel et al. showed that an EGFR antisense oligonucleotide treatment resulted in a significant reduction of VEGF protein expression, and addition of conditioned medium from EGFR antisense-treated tumor cells resulted in decreased endothelial cell migration [221]. The combination of bevacizumab (a humanized monoclonal IgG1 antibody targeting VEGF) with erlotinib was well tolerated and had a response rate of 15 % [222], which, in a cross trial comparison, was higher than the response rate for erlotinib alone (5 %) [185] or the VEGFR inhibitors SU5416 alone (5 %) [223] or sorafenib (an inhibitor of VEGFR, PDGFR, Raf kinase, and others) alone (3–4 %) [224]. Argiris et al. demonstrated that the combination of bevacizumab and cetuximab enhanced growth inhibition both in vivo and in vitro in preclinical models and resulted in a SCCHN disease control rate of 46 % [225]. Chemotherapy with

or without bevacizumab is being investigated in a phase III trial with patients with recurrent or metastatic head and neck cancer (NCT00588770).

A novel fully humanized dual-targeting IgG (DT-IgG) antibody that simultaneously targets VEGF and EGFR has been designed to optimize tumor targeting and maximize potential clinical benefits [226]. Hurwitz et al. tested DT-IgG on SCCHN, lung adenocarcinoma, and colon cancer xenograft models and discovered that DT-IgG had a lower in vivo IC_{50} than bevacizumab (VEGF targeting antibody) and cetuximab; however, a higher dose of DT-IgG was needed to produce efficacy similar to that observed with combined bevacizumab and cetuximab treatment [226]. Zang et al. showed in SCCHN in vitro studies that DT-IgG neutralizes VEGF as effectively as bevacizumab and inhibits EGFR activation and cell proliferation as effectively as cetuximab [227]. One obvious benefit of DT-IgG therapy would be avoidance of dosing complications associated with drug combinations [226], [227].

4.5.4 STAT3 Expression

Sen et al. found that increased STAT3 may contribute to cetuximab resistance in SCCHN [210]. STAT3 inhibition in cetuximab-resistant SCCHN cells using a STAT3 decoy oligonucleotide to inhibit STAT3-mediated transcription reduced cellular viability and the expression of STAT3 target genes. STAT3 decoy treatment also successfully decreased tumor growth in vivo [210].

4.5.5 KRAS Mutation

KRAS mutations are fairly rare in SCCHN compared to other types of cancer [228]. Mutational activation of KRAS only occurred in 2.6 % of 115 clinical specimens of SCCHN, although copy number amplification of KRAS was found in 10 samples (8.7 %) in the same study [229]. In another study, KRAS mutations were found in 4 out of 29 patients with SCCHN and the presence of the G12V KRAS mutation was associated with an absence of response to cetuximab and radiotherapy [230].

4.5.6 Microenvironment

A growing body of evidence suggests that components of the tumor microenvironment may also contribute to tumorigenesis in cancers of epithelial origin and may modulate the treatment sensitivity of tumor cells [231]. Johansson et al. reported that cancer-associated fibroblasts (CAFs) offered protection from cetuximab treatment and negate cetuximab-induced growth inhibition [213]. They further described that SCCHN cell lines cocultured with CAFs from patients with SCCHN resulted in elevated expression of matrix metalloproteinase-1 (MMP-1) in both the tumor cells

and the CAFs. MMP inhibitors can partly abolish the CAF-induced resistance; however, siRNA knockdown of MMP-1 in CAFs did not abolish resistance, suggesting that other MMP family members may be involved [213]. The mechanism of MMP associated cetuximab resistance is not clear and further investigation is warranted.

4.5.7 Epigenetic Changes

Emerging evidence has indicated connections between epigenetic changes, such as DNA methylation at CpG islands, and development of resistance to multiple cancer therapeutics [232]–[235]. Ogawa et al. tested a panel of 56 genes (including death-associated protein kinase (DAPK), MGMT, and SRBC), commonly known to be regulated through promoter methylation, using array-based methylation analysis of two parental NSCLC and SCCHN cell lines and subclones from those cell lines rendered resistant to either erlotinib or cetuximab. The study found that DAPK was hypermethylated in NSCLC and SCCHN drug-resistant cells. Subsequent demethylation of DAPK in the resistant NSCLC cells restored sensitivity to both erlotinib and cetuximab. siRNA-mediated knockdown of DAPK validated the array-based findings by inducing erlotinib and cetuximab resistance in cells normally sensitive to either agent [214].

4.5.8 Other Factors

Transforming growth factor beta (TGFβ) has recently been shown to be a key molecular determinant of *de novo* and acquired resistance of cancers to EGFR-targeted mAbs [236]. Bedi et al. found that treatment of mice bearing xenografts of human SCCHN cells with cetuximab resulted in emergence of resistant tumor cells that expressed relatively higher levels of TGFβ compared to the control group. Also, treatment with cetuximab alone induced an apparent natural selection of TGFβ-overexpressing tumor cells in nonregressing tumors. Combinatorial treatment with cetuximab and a TGFβ-blocking antibody prevented the emergence of resistant tumor cells and induced complete tumor regression [236].

Hoellein et al. showed in a sample of 180 patients that coexpression of elevated levels of Aurora-A and EGFR was an adverse prognostic factor with poor disease-free and overall survival [237]. Hoellein et al. further showed significant overexpression of Aurora-A and EGFR in SCCHN tumors compared to normal adjacent mucosa. In vitro studies showed that simultaneous targeting of Aurora kinase and EGFR using cetuximab and a pan-Aurora kinase inhibitor (R763) was more effective than mono-EGFR or mono-Aurora kinase inhibition. Interestingly, growth inhibitory effects were noticeable with the addition of R763 to cell lines with no or very moderate response to mono-EGFR-targeted treatment and/or with very low EGFR expression

[237]. Independent studies have shown efficacy of combination of a specific Aurora-A inhibitor with erlotinib or cetuximab in an EGFR-dependent cancer cell line [238]. These findings suggest that Aurora kinase inhibitors may help overcome cetuximab resistance in the treatment of SCCHN; however, more work is needed.

4.6 Conclusions and New Frontiers in Drug Discovery

Even though many ErbB-associated individual pathways have been identified as crucial in the development and progression of many cancers as well as in the resistance to EGFRIs, there remain many cases in which the exact mechanism of resistance is unclear. Systems biology can help guide the integration of information and data to gain insights into the interconnectedness of ErbB-associated pathways in the context of tumorigenesis and metastatic disease [239]. Systems biology approaches together with high-throughput screening presents a great opportunity to better understand buildup of resistance and to rationally investigate potentially new therapeutic combinations [240].

Astsaturov et al. recently used synthetic-lethal screening to probe an EGFR-centered network using erlotinib and nonspecific cytotoxic agents. In this study, Astsaturov et al. successfully identified a cluster of resistance-determining proteins, shedding light on, until then, underappreciated or completely novel interactions. It was also shown that pharmaceuticals targeting certain proteins (e.g., Aurora A, PKC, STAT3) in the network synergized with EGFRIs to markedly reduce cell proliferation and tumor size [238]. Synthetic lethal screening approaches clearly are a valuable tool available to coherently improve targeted therapies focused on EGFR inhibition.

Another valuable addition to the field of systems biology was recently made by Fertig et al. [241]. Fertig et al. developed a new method that provides gene expression patterns and biological process activity from transcriptomics data accessible through an add-on for the popular free statistical computing software R [242]. In their most recent paper, Fertig et al. further demonstrated that gene expression signatures can be successfully generated using their algorithm and that these output data are pertinent to downstream effects of EGFR in SCCHN and useful for modeling contributing elements to cetuximab resistance [243].

SCCHN and its treatment illustrate the basic paradigm that is likely to shape personalized medicine: identification and assessment of relevant genes (e.g., high EGFR expression); development of target-specific therapeutics (e.g., cetuximab); exploration of relevant signaling pathways (e.g., Ras/Raf/MEK); elucidation of mechanisms of resistance (e.g., VEGF expression); and redesign of therapies (e.g., dual inhibitors like lapatinib and antibodies targeting VEGF and EGFR simultaneously). It can be expected that within the next decade the scientific and medical community will be able to accurately analyze disease using a patient's complete genetic profile and tailor treatment approaches appropriately [244]. The Encyclopedia of DNA Elements (ENCODE) project has greatly enriched our understanding of the functional elements of the human genome, a fundamental stepping-stone for the advancement of personalized medicine in oncology [245], particularly considering

expanding complementary information on somatic mutations in cancer (COSMIC; [246]) and data sets of multidimensional cancer genomics (cBio Cancer Genomics Portal; [247]). A multitude of databases on protein–protein interactions and disease-associated signaling patterns already exist and can be used to build comprehensive signaling networks that highlight potential oncogenic hubs and allow rational hypothesis formulation and testing [248]. As the collective knowledge of genetics and proteomics increases and even more data become available, systems biology will continue to play an ever-increasing role in genetic interaction mapping, identification of pathway activation biomarkers, and elucidation of cancer-associated genes [239], possibly soon on an individual level.

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References

1. Murray, S, et al. Screening for EGFR mutations in patients with head and neck cancer treated with gefitinib on a compassionate-use program: a hellenic cooperative oncology group study. J Oncol. 2010;2010:709678.
2. Society AC. Cancer facts & figs. 2012. http://www.cancer.org. Accessed 1 July 2012.
3. Argiris A, et al. Head and neck cancer. Lancet. 2008;371(9625):1695–709.
4. Zimmermann M, et al. The epidermal growth factor receptor (EGFR) in head and neck cancer: its role and treatment implications. Radiat Oncol. 2006;1:11.
5. Pignon JP, et al. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC collaborative group. Meta-Analysis of chemotherapy on head and neck cancer. Lancet. 2000;355(9208):949–55.
6. Bouyain S, et al. The extracellular region of ErbB4 adopts a tethered conformation in the absence of ligand. Proc Natl Acad Sci U S A. 2005;102(42):15024–9.
7. Cho HS, Leahy DJ. Structure of the extracellular region of HER3 reveals an interdomain tether. Science. 2002;297(5585):1330–3.
8. Ferguson KM, et al. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol Cell. 2003;11(2):507–17.
9. Franklin MC, et al. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell. 2004;5(4):317–28.
10. Shi F, et al. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. Proc Natl Acad Sci U S A. 2010;107(17):7692–7.
11. Cho HS, et al. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature. 2003;421(6924):756–60.
12. Garrett TP, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell. 2003;11(2):495–505.
13. Bassiri M, Privalsky ML. Mutagenesis of the avian erythroblastosis virus erbB coding region: an intact extracellular domain is not required for oncogenic transformation. J Virol. 1986;59(2):525–30.
14. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res. 1993;53(15):3579–84.

15. Grandis JR, Tweardy DJ. TGF-alpha and EGFR in head and neck cancer. J Cell Biochem Suppl. 1993;17F:188–91.
16. Hitt R, et al. Prognostic value of the epidermal growth factor receptor (EGRF) and p53 in advanced head and neck squamous cell carcinoma patients treated with induction chemotherapy. Eur J Cancer. 2005;41(3):453–60.
17. Liccardi G, Hartley JA, Hochhauser D. EGFR nuclear translocation modulates DNA repair following cisplatin and ionizing radiation treatment. Cancer Res. 2011;71(3):1103–14.
18. Ang KK, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res. 2002;62(24):7350–6.
19. Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol. 2006;24(17):2666–72.
20. Rubin Grandis J, et al. Quantitative immunohistochemical analysis of transforming growth factor-alpha and epidermal growth factor receptor in patients with squamous cell carcinoma of the head and neck. Cancer. 1996;78(6):1284–92.
21. Shin DM, et al. Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. Cancer Res. 1994;54(12):3153–9.
22. O'Rorke MA, et al. Human papillomavirus related head and neck cancer survival: a systematic review and meta-analysis. Oral Oncol. 2012;48(12):1191–201.
23. Keller J, et al. Combination of phosphorylated and truncated EGFR correlates with higher tumor and nodal stage in head and neck cancer. Cancer Invest. 2010;28(10):1054–62.
24. Maurizi M, et al. Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. Br J Cancer. 1996;74(8):1253–7.
25. Chang AR, et al. Expression of epidermal growth factor receptor and cyclin D1 in pretreatment biopsies as a predictive factor of radiotherapy efficacy in early glottic cancer. Head Neck. 2008;30(7):852–7.
26. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol. 2010;10(5):317–27.
27. Rabinowits G, Haddad RI. Overcoming resistance to EGFR inhibitor in head and neck cancer: a review of the literature. Oral Oncol. 2012;48(11):1085–9.
28. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. Nat Rev Cancer. 2009;9(1):28–39.
29. Wilson KJ, et al. Functional selectivity of EGF family peptide growth factors: implications for cancer. Pharmacol Ther. 2009;122(1):1–8.
30. Burgess AW, et al. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell. 2003;12(3):541–52.
31. Garrett TP, et al. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. Cell. 2002;110(6):763–73.
32. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell. 2002;110(6):669–72.
33. Rogers SJ, et al. Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer Metastasis Rev. 2005;24(1):47–69.
34. Endres NF, et al. Regulation of the catalytic activity of the EGF receptor. Curr Opin Struct Biol. 2011;21(6):777–84.
35. Alvarado D, Klein DE, Lemmon MA. Structural basis for negative cooperativity in growth factor binding to an EGF receptor. Cell. 2010;142(4):568–79.
36. Jura N, et al. Catalytic control in the EGF receptor and its connection to general kinase regulatory mechanisms. Mol Cell. 2011;42(1):9–22.
37. Oksvold MP, et al. Serine mutations that abrogate ligand-induced ubiquitination and internalization of the EGF receptor do not affect c-Cbl association with the receptor. Oncogene. 2003;22(52):8509–18.
38. Oksvold MP, et al. UV-radiation-induced internalization of the epidermal growth factor receptor requires distinct serine and tyrosine residues in the cytoplasmic carboxy-terminal domain. Radiat Res. 2004;161(6):685–91.

39. Tong J, et al. Epidermal growth factor receptor phosphorylation sites Ser991 and Tyr998 are implicated in the regulation of receptor endocytosis and phosphorylations at Ser1039 and Thr1041. Mol Cell Proteomics. 2009;8(9):2131–44.
40. Orth JD, et al. A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. Cancer Res. 2006;66(7):3603–10.
41. Sigismund S, et al. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell. 2008;15(2):209–19.
42. Schroeder B, et al. A Dyn2-CIN85 complex mediates degradative traffic of the EGFR by regulation of late endosomal budding. Embo Journal. 2010;29(18):3039–53.
43. Brand TM, et al. The nuclear epidermal growth factor receptor signaling network and its role in cancer. Discov Med. 2011;12(66):419–32.
44. Wang YN, et al. COPI-mediated retrograde trafficking from the Golgi to the ER regulates EGFR nuclear transport. Biochem Biophys Res Commun. 2010;399(4):498–504.
45. Wang YN, et al. Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. Oncogene. 2010;29(28):3997–4006.
46. Liao HJ, Carpenter G. Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. Mol Biol Cell. 2007;18(3):1064–72.
47. Wang YN, Hung MC. Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. Cell Biosci. 2012;2:13.
48. Morgan S, Grandis JR. ErbB receptors in the biology and pathology of the aerodigestive tract. Exp Cell Res. 2009;315(4):572–82.
49. Bei R, et al. Co-localization of multiple ErbB receptors in stratified epithelium of oral squamous cell carcinoma. J Pathol. 2001;195(3):343–8.
50. Bei R, et al. Frequent overexpression of multiple ErbB receptors by head and neck squamous cell carcinoma contrasts with rare antibody immunity in patients. J Pathol. 2004;204(3): 317–25.
51. Ekberg T, et al. Expression of EGFR, HER2, HER3, and HER4 in metastatic squamous cell carcinomas of the oral cavity and base of tongue. Int J Oncol. 2005;26(5):1177–85.
52. Chung CH, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol. 2006;24(25):4170–6.
53. Rubin Grandis J, et al. Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. J Natl Cancer Inst. 1998;90(11):824–32.
54. Rajput A, et al. A novel mechanism of resistance to epidermal growth factor receptor antagonism in vivo. Cancer Res. 2007;67(2):665–73.
55. Jhappan C, et al. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell. 1990;61(6):1137–46.
56. Matsui Y, et al. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell. 1990;61(6):1147–55.
57. Sandgren EP, et al. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell. 1990;61(6):1121–35.
58. Tinhofer I, et al. Expression of amphiregulin and EGFRvIII affect outcome of patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment. Clin Cancer Res. 2011;17(15):5197–204.
59. Yonesaka K, et al. Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers. Clin Cancer Res. 2008;14(21):6963–73.
60. Hatakeyama H, et al. Regulation of heparin-binding EGF-like growth factor by miR-212 and acquired cetuximab-resistance in head and neck squamous cell carcinoma. Plos One. 2010;5(9):e12702.
61. O-Charoenrat P, Rhys-Evans P, Eccles S. Expression and regulation of c-erbB ligands in human head and neck squamous carcinoma cells. Int J Cancer. 2000;88(5):759–65.
62. Murakami H, et al. Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor alpha in hepatic oncogenesis. Cancer Res. 1993;53(8):1719–23.

63. Sandgren EP, et al. Transforming growth factor alpha dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. Mol Cell Biol. 1993;13(1):320–30.
64. Sandgren EP, et al. Inhibition of Mammary-Gland involution is associated with transforming Growth-Factor-Alpha but Not C-Myc-Induced tumorigenesis in transgenic mice. Cancer Res. 1995;55(17):3915–27.
65. Oshima G, et al. Autocrine epidermal growth factor receptor ligand production and cetuximab response in head and neck squamous cell carcinoma cell lines. J Cancer Res Clin Oncol. 2012;138(3):491–9.
66. Psyrri A, et al. Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. Clin Cancer Res. 2005;11(16):5856–62.
67. Psyrri A, et al. Correlates and determinants of nuclear epidermal growth factor receptor content in an oropharyngeal cancer tissue microarray. Cancer Epidemiology Biomarkers Prevention. 2008;17(6):1486–92.
68. Hama T, et al. Prognostic significance of epidermal growth factor receptor phosphorylation and mutation in head and neck squamous cell carcinoma. Oncologist. 2009;14(9):900–8.
69. Schwentner I, et al. Identification of the rare EGFR mutation p.G796S as somatic and germline mutation in white patients with squamous cell carcinoma of the head and neck. Head Neck. 2008;30(8):1040–4.
70. Lee JW, et al. Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. Clin Cancer Res. 2005;11(8):2879–82.
71. Stransky N, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60.
72. Agrawal N, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–7.
73. Diedrich U, et al. Distribution of epidermal growth factor receptor gene amplification in brain tumours and correlation to prognosis. J Neurol. 1995;242(10):683–8.
74. Garcia de Palazzo IE, et al. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res. 1993;53(14):3217–20.
75. Ge H, Gong X, Tang CK. Evidence of high incidence of EGFRvIII expression and coexpression with EGFR in human invasive breast cancer by laser capture microdissection and immunohistochemical analysis. Int J Cancer. 2002;98(3):357–61.
76. Moscatello DK, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res. 1995;55(23):5536–9.
77. Nishikawa R, et al. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc Natl Acad Sci U S A. 1994;91(16):7727–31.
78. Okamoto I, et al. Expression of constitutively activated EGFRvlll in non-small cell lung cancer. Cancer Sci. 2003;94(1):50–6.
79. Chau NG, et al. The association between EGFR variant III, HPV, p16, c-MET, EGFR gene copy number and response to EGFR inhibitors in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. Head Neck Oncol. 2011;3:11.
80. Sok JC, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res. 2006;12(17):5064–73.
81. Yang B, et al. Expression of epidermal growth factor receptor variant III in laryngeal carcinoma tissues. Auris Nasus Larynx. 2009;36(6):682–7.
82. McIntyre JB, et al. Specific and sensitive hydrolysis probe-based real-time PCR detection of epidermal growth factor receptor variant III in oral squamous cell carcinoma. Plos One. 2012;7(2):e31723.
83. Matta A, Ralhan R. Overview of current and future biologically based targeted therapies in head and neck squamous cell carcinoma. Head Neck Oncol. 2009;1:6.
84. Chang KY, et al. Novel phosphoinositide 3-kinase/mTOR dual inhibitor, NVP-BGT226, displays potent growth-inhibitory activity against human head and neck cancer cells in vitro and in vivo. Clin Cancer Res. 2011;17(22):7116–26.

85. Lai SY, Johnson FM. Defining the role of the JAK-STAT pathway in head and neck and thoracic malignancies: implications for future therapeutic approaches. Drug Resist Updates. 2010;13(3):67–78.
86. Wang SJ, Bourguignon LY. Hyaluronan-CD44 promotes phospholipase C-mediated Ca2 + signaling and cisplatin resistance in head and neck cancer. Arch Otolaryngol Head Neck Surg. 2006;132(1):19–24.
87. Cohen EE, et al. Protein kinase C zeta mediates epidermal growth factor-induced growth of head and neck tumor cells by regulating mitogen-activated protein kinase. Cancer Res. 2006;66(12):6296–303.
88. Stabile LP, He G, Lui VW, Henry C, Gubish CT, Joyce S, Quesnelle K, Siegfried JM, Grandis JR. c-Src activation mediates erlotinib resistance in head and neck cancer by stimulating c-Met. Clin Cancer Res. 2013 Jan 15;19(2):380–92.
89. Roskoski R Jr. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res. 2012;66(2):105–43.
90. Wortzel I, Seger R. The ERK cascade: distinct functions within various subcellular organelles. Genes Cancer. 2011;2(3):195–209.
91. Lurje G, Lenz HJ. EGFR signaling and drug discovery. Oncology. 2009;77(6):400–10.
92. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nature Reviews Drug Discovery. 2006;5(8):671–88.
93. Quesnelle KM, Boehm AL, Grandis JR. STAT-mediated EGFR signaling in cancer. Journal of Cellular Biochemistry. 2007;102(2):311–9.
94. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nature Reviews Cancer. 2009;9(11):798–809.
95. Mehra R, et al. Protein-intrinsic and signaling network-based sources of resistance to EGFR- and ErbB family-targeted therapies in head and neck cancer. Drug Resist Updat. 2011;14(6):260–79.
96. Hwang JI, et al. Molecular cloning and characterization of a novel phospholipase C, PLC-eta. Biochem J. 2005;389(Pt 1):181–6.
97. Thomas SM, et al. Epidermal growth factor receptor-stimulated activation of phospholipase Cgamma-1 promotes invasion of head and neck squamous cell carcinoma. Cancer Res. 2003;63(17):5629–35.
98. Kassis J, et al. A role for phospholipase C-gamma-mediated signaling in tumor cell invasion. Clin Cancer Res. 1999;5(8):2251–60.
99. Nozawa H, et al. Combined inhibition of PLC gamma-1 and c-Src abrogates epidermal growth factor receptor-mediated head and neck squamous cell carcinoma invasion. Clin Cancer Res. 2008;14(13):4336–44.
100. Rosse C, et al. PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol. 2010;11(2):103–12.
101. Martinezgimeno C, et al. Alterations in levels of different Protein-Kinase-C isotypes and their influence on behavior of Squamous-Cell carcinoma of the oral cavity—Epsilon-Pkc, a novel prognostic factor for relapse and survival. Head Neck. 1995;17(6):516–25.
102. Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. Oncogene. 2004;23(48):7906–9.
103. Koppikar P, et al. Combined inhibition of c-Src and epidermal growth factor receptor abrogates growth and invasion of head and neck squamous cell carcinoma. Clin Cancer Res. 2008;14(13):4284–91.
104. Egloff AM, Grandis JR. Improving response rates to EGFR-targeted therapies for head and neck squamous cell carcinoma: candidate predictive biomarkers and combination treatment with Src inhibitors. J Oncol. 2009;2009:896407.
105. Wheeler DL, Iida M, Dunn EF. The role of Src in solid tumors. Oncologist. 2009;14(7):667–78.
106. Xi S, et al. Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. J Biol Chem. 2003;278(34):31574–83.

107. Zhang Q, et al. SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. Cancer Res. 2004;64(17):6166–73.
108. Li C, et al. Nuclear EGFR contributes to acquired resistance to cetuximab. Oncogene. 2009;28(43):3801–13.
109. Yeatman TJ. A renaissance for SRC. Nat Rev Cancer. 2004;4(6):470–80.
110. Yan M, et al. Correlation of NF-kappa B signal pathway with tumor metastasis of human head and neck squamous cell carcinoma. Bmc Cancer. 2010;10:437.
111. Ferris RL, Grandis JR. NF-kappa B gene signatures and p53 mutations in head and neck squamous cell carcinoma—Commentary. Clin Cancer Res. 2007;13(19):5663–4.
112. Nakayama H, et al. High expression levels of nuclear factor kappa B, I kappa B kinase alpha and Akt kinase in squamous cell carcinoma of the oral cavity. Cancer. 2001;92(12):3037–44.
113. Yan B, et al. Genome-wide identification of novel expression signatures reveal distinct patterns and prevalence of binding motifs for p53, nuclear factor-kappaB and other signal transcription factors in head and neck squamous cell carcinoma. Genome Biol. 2007;8(5):R78.
114. Arun P, et al. Nuclear NF-kappa B p65 phosphorylation at serine 276 by Protein kinase a contributes to the malignant phenotype of head and neck cancer. Clin Cancer Res. 2009;15(19):5974–84.
115. Tanaka T, et al. Selective inhibition of nuclear factor-?B by nuclear factor-?B essential modulator-binding domain peptide suppresses the metastasis of highly metastatic oral squamous cell carcinoma. Cancer Sci. 2012;103(3):455–63.
116. Aravindan N, et al. Irreversible EGFR inhibitor EKB-569 Targets Low-LET gamma-Radiation-Triggered rel orchestration and potentiates cell death in squamous cell carcinoma. Plos One. 2011; 6(12):e29705.
117. Wilken R, et al. Curcumin: a review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Molecular Cancer. 2011;10:12.
118. Lee TL, et al. A signal network involving coactivated NF-kappa B and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas. Int J Cancer. 2008;122(9):1987–98.
119. Bivona TG, et al. FAS and NF-kappaB signalling modulate dependence of lung cancers on mutant EGFR. Nature. 2011;471(7339):523–6.
120. Calais G, et al. Randomized trial of radiation therapy versus concomitant chemotherapy and radiation therapy for advanced-stage oropharynx carcinoma. J Natl Cancer Inst. 1999;91(24):2081–6.
121. Adelstein DJ, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. J Clin Oncol. 2003;21(1):92–8.
122. Trotti A, et al. TAME: development of a new method for summarising adverse events of cancer treatment by the Radiation Therapy Oncology Group. Lancet Oncol. 2007;8(7):613–24.
123. Dittmann K, et al. Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. J Biol Chem. 2005;280(35):31182–9.
124. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol. 2003;21(14):2787–99.
125. Vermorken JB, et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol. 2007;25(16):2171–7.
126. Dent P, et al. Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. Mol Biol Cell. 1999;10(8):2493–506.
127. Schmidt-Ullrich RK, et al. Altered expression of epidermal growth factor receptor and estrogen receptor in MCF-7 cells after single and repeated radiation exposures. Int J Radiat Oncol Biol Phys. 1994;29(4):813–9.

128. Bonner JA, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006;354(6):567–78.
129. Numico G, Franco P, Cristofano A, Migliaccio F, Spinazzé S, Silvestris N, Cante D, Sciacero P, La Porta MR, Girelli F, Ricardi U. Is the combination of cetuximab with chemo-radiotherapy regimens worthwhile in the treatment of locally advanced head and neck cancer? A review of current evidence. Crit Rev Oncol Hematol. 2013 Feb;85(2):112–20.
130. Fakih M, Vincent M. Adverse events associated with anti-EGFR therapies for the treatment of metastatic colorectal cancer. Curr Oncol. 2010;17(Suppl 1):S18–30.
131. Moon C, Chae YK, Lee J. Targeting epidermal growth factor receptor in head and neck cancer: lessons learned from cetuximab. Exp Biol Med (Maywood). 2010;235(8):907–20.
132. Gerber PA, et al. Management of EGFR-inhibitor associated rash: a retrospective study in 49 patients. Eur J Med Res. 2012;17:4.
133. Sipples R. Common side effects of anti-EGFR therapy: acneform rash. Semin Oncol Nurs. 2006;22(1 Suppl 1):28–34.
134. Vermorken JB, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116–27.
135. Masui H, et al. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res. 1984;44(3):1002–7.
136. Wheeler DL, Dunn EF, Harari PM. Understanding resistance to EGFR inhibitors-impact on future treatment strategies. Nat Rev Clin Oncol. 2010;7(9):493–507.
137. Mendelsohn J. Blockade of receptors for growth factors: an anticancer therapy–the fourth annual Joseph H Burchenal American Association of Cancer Research Clinical Research Award Lecture. Clin Cancer Res. 2000;6(3):747–53.
138. Specenier P, Vermorken JB. Biologic therapy in head and neck cancer: a road with hurdles. ISRN Oncol. 2012;2012:163752.
139. Schmitz KR, Ferguson KM. Interaction of antibodies with ErbB receptor extracellular regions. Exp Cell Res. 2009;315(4):659–70.
140. Marshall J. Clinical implications of the mechanism of epidermal growth factor receptor inhibitors. Cancer. 2006;107(6):1207–18.
141. Kurai J, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clin Cancer Res. 2007;13(5):1552–61.
142. Mehra R, Cohen RB, Burtness BA. The role of cetuximab for the treatment of squamous cell carcinoma of the head and neck. Clin Adv Hematol Oncol. 2008;6(10):742–50.
143. Trigo J, et al. Cetuximab monotherapy is active in patients (pts) with platinum-refractory recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN): results of a phase II study. J Clin Oncol. 2004;22:488s.
144. Baselga J, et al. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. J Clin Oncol. 2000;18(4):904–14.
145. Robert F, et al. Phase I study of anti–epidermal growth factor receptor antibody cetuximab in combination with radiation therapy in patients with advanced head and neck cancer. J Clin Oncol. 2001;19(13):3234–43.
146. Bonner JA, et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol. 2010;11(1):21–8.
147. Merlano M, Occelli M. Review of cetuximab in the treatment of squamous cell carcinoma of the head and neck. Ther Clin Risk Manag. 2007;3(5):871–6.
148. Burtness B, et al. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005;23(34):8646–54.
149. Licitra L, Storkel S, Kerr KM, Van Cutsem E, Pirker R, Hirsch FR, Vermorken JB, von Heydebreck A, Esser R, Celik I, Ciardiello F. Predictive value of epidermal growth factor receptor expression for first-line chemotherapy plus cetuximab in patients with head and neck and colorectal cancer: analysis of data from the extreme and crystal studies. Eur J Cancer. 2013 April;49(6):1161–8.

150. Nielsen DL, Pfeiffer P, Jensen BV. Six cases of treatment with panitumumab in patients with severe hypersensitivity reactions to cetuximab. Ann Oncol. 2009;20(4):798.
151. Wirth LJ, et al. Phase I dose-finding study of paclitaxel with panitumumab, carboplatin and intensity-modulated radiotherapy in patients with locally advanced squamous cell cancer of the head and neck. Ann Oncol. 2010;21(2):342–7.
152. Machiels JP, et al. 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: an open-label, randomised phase 3 trial. Lancet Oncol. 2011;12(4):333–43.
153. Mateo C, et al. Humanization of a mouse monoclonal antibody that blocks the epidermal growth factor receptor: recovery of antagonistic activity. Immunotechnology. 1997;3(1): 71–81.
154. Rivera F, et al. Current situation of Panitumumab, Matuzumab, Nimotuzumab and Zalutumumab. Acta Oncol. 2008;47(1):9–19.
155. Rojo F, et al. Pharmacodynamic trial of nimotuzumab in unresectable squamous cell carcinoma of the head and neck: a SENDO Foundation study. Clin Cancer Res. 2010;16(8):2474–82.
156. Rodriguez MO, et al. Nimotuzumab plus radiotherapy for unresectable squamous-cell carcinoma of the head and neck. Cancer Biol Ther. 2010;9(5):343–9.
157. Schmiedel J, et al. Matuzumab binding to EGFR prevents the conformational rearrangement required for dimerization. Cancer Cell. 2008;13(4):365–73.
158. Rao S, et al. Phase I study of epirubicin, cisplatin and capecitabine plus matuzumab in previously untreated patients with advanced oesophagogastric cancer. Br J Cancer. 2008;99(6):868–74.
159. Rao S, et al. Matuzumab plus epirubicin, cisplatin and capecitabine (ECX) compared with epirubicin, cisplatin and capecitabine alone as first-line treatment in patients with advanced oesophago-gastric cancer: a randomised, multicentre open-label phase II study. Ann Oncol. 2010;21(11):2213–9.
160. Yonesaka K, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med. 2011;3(99):99ra86.
161. Gutierrez VF, et al. Genetic profile of second primary tumors and recurrences in head and neck squamous cell carcinomas. Head Neck. 2012;34(6):830–9.
162. Lange T, et al. Trastuzumab has anti-metastatic and anti-angiogenic activity in a spontaneous metastasis xenograft model of esophageal adenocarcinoma. Cancer Lett. 2011;308(1):54–61.
163. Norman G, et al. Trastuzumab for the treatment of HER2-positive metastatic adenocarcinoma of the stomach or gastro-oesophageal junction. Health Technol Assess. 2011;15(Suppl 1): 33–42.
164. Slamon DJ, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344(11):783–92.
165. Bang YJ, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010;376(9742):687–97.
166. Kondo N, et al. Antitumor effect of gefitinib on head and neck squamous cell carcinoma enhanced by trastuzumab. Oncol Rep. 2008;20(2):373–8.
167. Kondo N, et al. Combined molecular targeted drug therapy for EGFR and HER-2 in head and neck squamous cell carcinoma cell lines. Int J Oncol. 2012;40(6):1805–12.
168. Wilson TR, et al. Neuregulin-1-mediated autocrine signaling underlies sensitivity to HER2 kinase inhibitors in a subset of human cancers. Cancer Cell. 2011;20(2):158–72.
169. Adams CW, et al. Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. Cancer Immunol Immunother. 2006;55(6):717–27.
170. Schoeberl B, et al. An ErbB3 antibody, MM-121, Is active in cancers with ligand-dependent activation. Cancer Res. 2010;70(6):2485–94.

171. Schoeberl B, et al. Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis. Sci Signal. 2009;2(77):ra31.
172. Robinson MK, et al. Targeting ErbB2 and ErbB3 with a bispecific single-chain Fv enhances targeting selectivity and induces a therapeutic effect in vitro. Br J Cancer. 2008;99(9):1415–25.
173. McDonagh CF, et al. Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. Mol Cancer Ther. 2012;11(3):582–93.
174. Hollmen M, et al. Suppression of breast cancer cell growth by a monoclonal antibody targeting cleavable ErbB4 isoforms. Oncogene. 2009;28(10):1309–19.
175. Vexler A, et al. Anti-ERBb4 targeted therapy combined with radiation therapy in prostate cancer. Results of in vitro and in vivo studies. Cancer Biol Ther. 2008;7(7):1090–4.
176. Agulnik M. New approaches to EGFR inhibition for locally advanced or metastatic squamous cell carcinoma of the head and neck (SCCHN). Med Oncol. 2012;29(4):2481–91.
177. Cohen EE, et al. Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol. 2003;21(10):1980–7.
178. Cohen EE, et al. Phase II trial of gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. Clin Cancer Res. 2005;11(23):8418–24.
179. Caponigro F, et al. A phase I/II trial of gefitinib and radiotherapy in patients with locally advanced inoperable squamous cell carcinoma of the head and neck. Anticancer Drugs. 2008;19(7):739–44.
180. Hainsworth JD, et al. Neoadjuvant chemotherapy/gefitinib followed by concurrent chemotherapy/radiation therapy/gefitinib for patients with locally advanced squamous carcinoma of the head and neck. Cancer. 2009;115(10):2138–46.
181. Stewart JS, et al. Phase III study of gefitinib compared with intravenous methotrexate for recurrent squamous cell carcinoma of the head and neck [corrected]. J Clin Oncol. 2009;27(11):1864–71.
182. Baselga J, et al. Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. J Clin Oncol. 2002;20(21):4292–302.
183. Perez CA, et al. Phase II study of gefitinib adaptive dose escalation to skin toxicity in recurrent or metastatic squamous cell carcinoma of the head and neck. Oral Oncol. 2012;48(9):887–92.
184. Argiris A, et al. A phase III randomized, placebo-controlled trial of docetaxel with or without gefitinib in recurrent or metastatic head and neck cancer: an Eastern Cooperative Oncology Group Trial. J Clin Oncol. 2013. (in press).
185. Soulieres D, et al. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. J Clin Oncol. 2004;22(1):77–85.
186. Siu LL, et al. Phase I/II trial of erlotinib and cisplatin in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: a princess margaret hospital phase II consortium and National Cancer Institute of Canada clinical trials group study. J Clin Oncol. 2007;25(16):2178–83.
187. Xia W, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene. 2002;21(41):6255–63.
188. de Souza JA, et al. A phase II study of lapatinib in recurrent/metastatic squamous cell carcinoma of the head and neck. Clin Cancer Res. 2012;18(8):2336–43.
189. Solca F, et al. Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker. J Pharmacol Exp Ther. 2012;343(2):342–50.
190. Markovic A, Chung CH. Current role of EGF receptor monoclonal antibodies and tyrosine kinase inhibitors in the management of head and neck squamous cell carcinoma. Expert Rev Anticancer Ther. 2012;12(9):1149–59.
191. Kalous O, et al. Dacomitinib (PF-00299804), an irreversible Pan-HER inhibitor, inhibits proliferation of HER2-amplified breast cancer cell lines resistant to trastuzumab and lapatinib. Mol Cancer Ther. 2012;11(9):1978–87.

192. Engelman JA, et al. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. Cancer Res. 2007;67(24):11924–32.
193. Abdul Razak AR, Soulieres D, Laurie SA, Hotte SJ, Singh S, Winquist E, Chia S, Le Tourneau C, Nguyen-Tan PF, Chen EX, Chan KK, Wang T, Giri N, Mormont C, Quinn S, Siu LL. A phase II trial of dacomitinib, an oral pan-human EGF receptor (HER) inhibitor, as first-line treatment in recurrent and/or metastatic squamous-cell carcinoma of the head and neck. Ann Oncol. 2013 Mar;24(3):761–9.
194. Cai X, et al. Discovery of 7-(4-(3-Ethynylphenylamino)-7-methoxyquinazolin-6-yloxy)-N-hydroxyheptanamide (CUDC-101) as a Potent Multi-Acting HDAC, EGFR, and HER2 inhibitor for the treatment of cancer. J Med Chem. 2010;53(5):2000–9.
195. Lai CJ, et al. CUDC-101, a multitargeted inhibitor of histone deacetylase, epidermal growth factor receptor, and human epidermal growth factor receptor 2, exerts potent anticancer activity. Cancer Res. 2010;70(9):3647–56.
196. Curran D, et al. Quality of life in head and neck cancer patients after treatment with high-dose radiotherapy alone or in combination with cetuximab. J Clin Oncol. 2007;25(16):2191–7.
197. Pryor DI, et al. Enhanced toxicity with concurrent cetuximab and radiotherapy in head and neck cancer. Radiother Oncol. 2009;90(2):172–6.
198. Burtness B, et al. NCCN task force report: management of dermatologic and other toxicities associated with EGFR inhibition in patients with cancer. J Natl Compr Canc Netw. 2009;7(Suppl 1):S5–21; quiz S22–4.
199. O'Neil BH, et al. High incidence of cetuximab-related infusion reactions in Tennessee and North Carolina and the association with atopic history. J Clin Oncol. 2007;25(24):3644–8.
200. Baas JM, et al. Recommendations on management of EGFR inhibitor-induced skin toxicity: a systematic review. Cancer Treat Rev. 2012;38(5):505–14.
201. Segaert S, Van Cutsem E. Clinical signs, pathophysiology and management of skin toxicity during therapy with epidermal growth factor receptor inhibitors. Ann Oncol. 2005;16(9):1425–33.
202. Gerber PA, et al. Therapy with epidermal growth factor receptor inhibitors. Hautarzt. 2010;61(8):654–61.
203. Wacker B, et al. Correlation between development of rash and efficacy in patients treated with the epidermal growth factor receptor tyrosine kinase inhibitor erlotinib in two large phase III studies. Clin Cancer Res. 2007;13(13):3913–21.
204. Saltz LB, et al. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol. 2004;22(7):1201–8.
205. Wheeler DL, et al. Mechanisms of acquired resistance to cetuximab: role of HER (ErbB) family members. Oncogene. 2008;27(28):3944–56.
206. Wheeler DL, et al. Epidermal growth factor receptor cooperates with Src family kinases in acquired resistance to cetuximab. Cancer Biol Ther. 2009;8(8):696–703.
207. Lu Y, et al. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the Anti-EGFR monoclonal antibody cetuximab. Cancer Res. 2007;67(17):8240–7.
208. Erjala K, et al. Signaling via ErbB2 and ErbB3 associates with resistance and epidermal growth factor receptor (EGFR) amplification with sensitivity to EGFR inhibitor gefitinib in head and neck squamous cell carcinoma cells. Clin Cancer Res. 2006;12(13):4103–11.
209. O-charoenrat P, et al. Vascular endothelial growth factor family members are differentially regulated by c-erbB signaling in head and neck squamous carcinoma cells. Clin Exp Metastasis. 2000;18(2):155–61.
210. Sen M, et al. Targeting Stat3 abrogates EGFR inhibitor resistance in cancer. Clin Cancer Res. 2012;18(18):4986–96.
211. Misale S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486(7404):532–6.
212. Diaz LA Jr, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537–40.

213. Johansson AC, et al. Cancer-associated fibroblasts induce matrix metalloproteinase-mediated cetuximab resistance in head and neck squamous cell carcinoma cells. Mol Cancer Res. 2012;10(9):1158–68.
214. Ogawa T, et al. Methylation of death-associated protein kinase is associated with cetuximab and erlotinib resistance. Cell Cycle. 2012;11(8):1656–63.
215. Ahsan A, et al. Role of epidermal growth factor receptor degradation in cisplatin-induced cytotoxicity in head and neck cancer. Cancer Res. 2010;70(7):2862–9.
216. Ritter CA, et al. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. Clin Cancer Res. 2007;13(16):4909–19.
217. Cabebe E, Wakelee H. Role of anti-angiogenesis agents in treating NSCLC: focus on bevacizumab and VEGFR tyrosine kinase inhibitors. Curr Treat Options Oncol. 2007;8(1):15–27.
218. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. Nature Reviews Cancer. 2008;8(8):579–91.
219. Ratushny V, et al. Targeting EGFR resistance networks in head and neck cancer. Cell Signal. 2009;21(8):1255–68.
220. Seiwert TY, Cohen EEW. Targeting angiogenesis in head and neck cancer. Semin Oncol. 2008;35(3):274–85.
221. Riedel F, et al. EGFR antisense treatment of human HNSCC cell lines down-regulates VEGF expression and endothelial cell migration. Int J Oncol. 2002;21(1):11–6.
222. Cohen EE, et al. Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study. Lancet Oncol. 2009;10(3):247–57.
223. Fury MG, et al. A phase II study of SU5416 in patients with advanced or recurrent head and neck cancers. Invest New Drugs. 2007;25(2):165–72.
224. Elser C, et al. Phase II trial of sorafenib in patients with recurrent or metastatic squamous cell carcinoma of the head and neck or nasopharyngeal carcinoma. J Clin Oncol. 2007;25(24):3766–73.
225. Argiris A, Kotsakis AP, Hoang T, Worden FP, Savvides P, Gibson MK, Gyanchandani R, Blumenschein GR Jr., Chen HX, Grandis JR, Harari PM, Kies MS, Kim S. Cetuximab and bevacizumab: preclinical data and phase ii trial in recurrent or metastatic squamous cell carcinoma of the head and neck. Ann Oncol. 2013 Jan;24(1):220–5.
226. Hurwitz SJ, et al. Pharmacodynamics of DT-IgG, a dual-targeting antibody against VEGF-EGFR, in tumor xenografted mice. Cancer Chemother Pharmacol. 2012;69(3):577–90.
227. Zhang H, et al. A dual-targeting antibody against EGFR-VEGF for lung and head and neck cancer treatment. Int J Cancer. 2012;131(4):956–69.
228. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008;9(7):517–31.
229. Suda T, et al. Copy number amplification of the PIK3CA gene is associated with poor prognosis in non-lymph node metastatic head and neck squamous cell carcinoma. Bmc Cancer. 2012;12(1):416.
230. Smilek P, et al. Epidermal growth factor receptor (EGFR) expression and mutations in the EGFR signaling pathway in correlation with anti-EGFR therapy in head and neck squamous cell carcinomas. Neoplasma. 2012;59(5):508–15.
231. McAllister SS, Weinberg RA. Tumor-host interactions: a far-reaching relationship. J Clin Oncol. 2010;28(26):4022–8.
232. Baylin SB, et al. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet. 2001;10(7):687–92.
233. Chang X, et al. Identification of hypermethylated genes associated with cisplatin resistance in human cancers. Cancer Res. 2010;70(7):2870–9.
234. Gifford G, et al. The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res. 2004;10(13):4420–6.
235. Segura-Pacheco B, et al. Global DNA hypermethylation-associated cancer chemotherapy resistance and its reversion with the demethylating agent hydralazine. J Transl Med. 2006;4:32.

236. Bedi A, et al. Inhibition of TGF-beta enhances the in vivo antitumor efficacy of EGF receptor-targeted therapy. Mol Cancer Ther. 2012;11(11):2429–39.
237. Hoellein A, et al. Aurora kinase inhibition overcomes cetuximab resistance in squamous cell cancer of the head and neck. Oncotarget. 2011;2(8):599–609.
238. Astsaturov I, et al. Synthetic lethal screen of an EGFR-centered network to improve targeted therapies. Sci Signal. 2010;3(140):ra67.
239. Chuang HY, Hofree M, Ideker T. A decade of systems biology. Annu Rev Cell Dev Biol. 2010;26:721–44.
240. Kohl P, et al. Systems biology: an approach. Clin Pharmacol Ther. 2010;88(1):25–33.
241. Fertig EJ, et al. CoGAPS: an R/C + + package to identify patterns and biological process activity in transcriptomic data. Bioinformatics. 2010;26(21):2792–3.
242. Team RDC. An introduction to R: notes on R, a programming environment for data analysis and graphics. Ockerbloom JM, editor. 2008.
243. Fertig EJ, et al. Gene expression signatures modulated by epidermal growth factor receptor activation and their relationship to cetuximab resistance in head and neck squamous cell carcinoma. Bmc Genomics. 2012;13:160.
244. Cordero P, Ashley EA. Whole-genome sequencing in personalized therapeutics. Clin Pharmacol Ther. 2012;91(6):1001–9.
245. Dunham I, et al. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489(7414):57–74.
246. Forbes SA, et al. COSMIC: mining complete cancer genomes in the catalogue of somatic mutations in cancer. Nucleic Acids Res. 2011;39(Database issue):D945–50.
247. Cerami E, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012;2(5):401–4.
248. Liu H, Beck TN, Golemis EA, Serebriiskii IG. Integrating in silico resources to map a signaling network. In: Ochs M, editor. Methods. New York: Springer; 2013.
249. Ogiso H, et al. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell. 2002;110(6):775–87.
250. Stamos J, Sliwkowski MX, Eigenbrot C. Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem. 2002;277(48):46265–72.
251. Zhang X, et al. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell. 2006;125(6):1137–49.
252. Bocharov EV, et al. Spatial structure of the dimeric transmembrane domain of the growth factor receptor ErbB2 presumably corresponding to the receptor active state. J Biol Chem. 2008;283(11):6950–6.
253. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5(5):341–54.

Chapter 5
The Role of HGF/c-MET in Head and Neck Squamous Cell Carcinoma

Tanguy Y. Seiwert, Tim N. Beck and Ravi Salgia

Abstract Head and Neck Squamous Cell Carcinoma (HNSCC) remains a formidable challenge to physicians, scientists, and patients. New targets that can be exploited to improve the outcome of patients afflicted with this dreadful disease are desperately needed: one such potential target is c-MET. The c-MET receptor tyrosine kinase, also known as hepatocyte growth factor receptor (HGFR), is robustly overexpressed and sometimes mutated or amplified in head and neck cancer cells, while overexpression of its ligand, hepatocyte growth factor/scatter factor (HGF/SF), often occurs in tumor-adjacent mesenchymal cells, providing paracrine signals that support tumor growth. Activation of c-MET stimulates numerous downstream signaling pathways that contribute to tumor growth, including GRB2/RAS, PI3K, STAT3, SRC, β-catenin, and Notch. Overexpression or anomalous activation of c-MET is often associated with resistance to targeted therapies inhibiting receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), that communicate to similar growth factor cascades. In this review, we emphasize the role of c-MET/HGF in HNSCC as well as the potential for therapeutic targeting of this receptor.

Keywords c-MET · HGFR · HGF · EGFR · HER · Head and neck cancer · Squamous cell cancer · Receptor tyrosine kinases · Targeted therapy

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5.1 Introduction

The biological behavior of head and neck squamous cell carcinomas (HNSCC) is similar to that of other squamous cell carcinomas (SCC), and is characterized by an aggressive growth pattern, tumor heterogeneity, early metastatic spread to lymph nodes, initial radiosensitivity and chemosensitivity as well as universally poor outcome once the disease spreads beyond the locoregional lymph node stations [1]–[3]. The underlying factors mediating the biological behavior of malignant pathologies frequently relate to the mechanisms transmitting extracellular signals to intracellular compartments. Such signaling pathways are critical for cell survival, growth, and metastasis. Better understanding of the molecular signaling pathways in HNSCC has provided a substantial opportunity for novel and individualized treatment in patients with these cancers [1].

While for many years the study of epidermal growth factor receptor (EGFR) signaling and development of EGFR targeting strategies predominated in HNSCC (discussed in Chap. 3), more recently the hepatocyte growth factor receptor c-MET and its ligand hepatocyte growth factor (HGF) have been found to play an important role in the pathogenesis of this disease [4], [5]. c-MET mediates multiple cellular processes, including survival, proliferation, and motility [6]–[9], and elevated expression of this protein in HNSCC has been associated with worse prognosis [5], [10], [11]. Elevated c-MET signaling also amplifies cross-talk with additional molecular pathways activated in HNSCC and other solid cancers, contributing to enhanced angiogenesis, tumor metabolism, survival, migration, and invasion [2], [12], [13].

In vitro studies performed with head and neck cancer (HNC) cell lines, and in vivo studies focused on other cancer types, have shown that c-MET inhibition can enhance the impact of established therapies, including combination therapies involving EGFR inhibitors or chemotherapeutics [14]. Although the clinical data for targeting c-MET are less mature for HNSCC than they are for other cancers (e.g., non-small cell lung cancer (NSCLC)), limited study results [15] and several highly relevant preclinical studies [11], [14], [16], [17] strongly suggest that disruption of c-MET signaling may be a promising approach for treating HNSCC, especially in combination with other therapeutics. This chapter summarizes what is known regarding normal and aberrant signaling of c-MET in HNSCC, and assesses the promise of HGF/c-MET targeting as a novel treatment strategy.

5.2 c-MET (Hepatocyte Growth Factor Receptor) Structure and Function

The c-MET tyrosine kinase receptor was initially discovered as the product of the oncogene translocated promoter region (TPR)-MET in a human osteogenic sarcoma cell line treated with the carcinogen N-Methyl-N’-nitro-N-nitrosoguanidine [18], [19]. The translocation involves a chromosomal rearrangement fusing TPR (encoding

a dimerization leucine zipper motif) from chromosome 1q25 and c-MET from chromosome 7q31 [20]. The normal c-MET gene encodes a single precursor amino acid chain; two transcript variants have been identified, one coding for 1,408 amino acids (isoform a) and the other coding for 1,390 amino acids (isoform b) [21]–[23]. Both isoforms have the same N- and C-termini. The c-MET signaling axis is functional in normal cells as well as in cancer (Fig. 5.1) [6], [7], [24].

5.2.1 c-MET General Structure

While c-MET is synthesized as a single chain of amino acids, it is posttranslationally cleaved by the endoprotease furin into α- and β-subunits as it passes through the endoplasmic reticulum [25–29]. The two subunits form an α-β heterodimer with a molecular weight of 190 kDa: a 50 kDa α-chain that is entirely extracellular, linked via a disulfide bond with the extracellular part of a 145 kDa β-chain [30], [31]. The β-chain entails a single pass transmembrane region and three major cytoplasmic domains (Fig. 5.1). Binding of the specific c-MET ligand hepatocyte growth factor/scatter factor (HGF/SF) occurs at the SEMA domain, which corresponds to part of the extracellular component of the β-chain aligned with and bound to the α-subunit. The SEMA domain has significant homology with other members of the semaphorin superfamily, which comprises c-MET, semaphorins, plexins, and Recepteur d'origine nantai (RON) [32].

The extracellular domain of the c-MET β-chain also includes immunoglobulin-like regions in plexins and transcription factor (IPT) domains. In vitro studies have suggested that the IPT domains, particularly IPT 3 and IPT 4 (Fig. 5.1), are necessary for high affinity binding of the α-chain of HGF, and that HGF binding to IPT domains can initiate signaling even in the absence of the SEMA domain; however, specific sensitivity to the activated form of HGF is lost in the absence of SEMA [33]. Within the cell, the β-chain encompasses a transmembrane domain, a juxtamembrane (JM) domain and a functional tyrosine kinase domain. Binding to HGF induces homodimerization of c-MET, and activates the intracellular kinase domain to induce trans-autophosphorylation of the cytoplasmic tail on Y1234 and Y1235, initiating downstream signaling events via the recruitment of effector proteins (Fig. 5.1) [34].

c-MET also heterodimerizes with the semaphorin superfamily member RON [35]. Macrophage-stimulating protein (MSP, also known as HGF-like protein) is the specific ligand for RON, and is secreted as pro-MSP prior to proteolytic activation by hepsin, membrane type serine protease 1 (MT-SP1 or matriptase) or HGF activator (HGFA) [36]. Whereas HGF is expressed in various tissue types, MSP is predominantly produced by liver cells [37]; however, MSP has been observed to play a relevant role in nonhepatic cancers, though no such observation has yet been made in HNCs [38]. RON itself on the other hand, like c-MET, has been implicated in HNSCC [39], as well as several other cancers, and presents another potential therapeutic target [40].

Fig. 5.1 HGF/c-MET structure, signaling and cross-talk hepatocyte growth factor/scatter factor (*HGF* or HGF/SF), a member of the plasminogen-related growth factor family, is secreted as a precursor (*pro-HGF*) by stromal cells (it can also be secreted in an autocrine fashion [137]) and is cleaved extracellularly by proteases. Once cleaved, HGF is converted to an active α- and β-chain heterodimer containing six domains: hairpin loop (*HL*; amino-terminal); kringle domains 1–4 (*K1–K4*), which are distinguishable by three conserved disulfide bonds; and the single β-chain domain, a serine protease homology (*SPH*) domain, which does not have proteolytic activity. c-MET is shown in its homodimeric form, consisting of a pair of α- and β-chains linked via a disulphide bond. The β-chain is a single pass transmembrane chain, with nine distinct domains: docking site (*DS*); catalytic region (*CR*); juxtamembrane sequence (*JM*); *i*mmunoglobulin-like fold shared by *p*lexins and transcriptional factors (*IPT1–4*); *p*lexins, *s*emaphorins and *i*ntegrins (*PSI;* domain is present in all three classes); and semaphoring (*SEMA*) domain. The SEMA domain consists of the entire α-chain and the extracellular N-terminus of the β-chain. The HGF β-chain binds to the SEMA domain, as shown; there is also evidence from in vitro studies that the HGF α-chain binds to IPT3 and IPT4 [138], [139]. The three c-MET intracellular regions, JM, CR and DS, all play

5.2.2 c-MET Trafficking

Endosomal compartmentalization and receptor trafficking have emerged as vital components of c-MET/HGF signaling [41]. Two c-MET-associated signaling effector proteins are significantly impacted by c-MET trafficking: signal transducer and activator of transcription 3 (STAT3) [41] and ERK1/2 [42]. c-MET endocytosis is controlled by clathrin-dependent and by clathrin-independent pathways [43]. Even though not all aspects of c-MET trafficking have been elucidated thus far, it has been shown that c-MET internalization can be mediated by PKC [42], Cbl [44], clathrin [45], dynamin 2 [46], sorting nexin 2 (SNX2) [47] and the Rho, Rac, and Rab GTPases [48], as well as presumably other mechanisms [43]. Endosomal compartmentalization provides spatiotemporal control over c-MET dependent signaling, allowing response specificity and fine-tuning of molecular events [49]. Altered profiles of c-MET endocytosis have been implicated in tumorigenesis of several cancers [43], [49], [50]; however, in the case of HNSCC, this aspect has not been adequately studied.

5.2.3 c-MET Ligands: HGF/SF

Although initial studies proposed the existence of two independent c-MET ligands, subsequent studies revealed that HGF and SF represent a single protein [25]. Similar to c-MET, HGF/SF is synthesized in an inactive single-chain propeptide form that requires proteolytic cleavage at residues Arg^{494}-Val^{495} [33]. In the case of HGF/SF, the proteases of relevance are matriptase (ST14), hepsin, and HGFA, which are required for HGF/SF to realize its biologically active heterodimeric form. Two known biological inhibitors of HGF/SF activation are HGF activator inhibitor 1 (HAI1) and HAI2 [25]. Structurally, the active form of HGF/SF is composed of a 68 kDa α-chain and a 34 kDa β-chain [28], [29], [51]. HGF/SF has a domain structure related to plasminogen—highlighting the link of HGF/SF to the coagulation cascade [52], which is of relevance in regenerative processes—and other serine proteinases; however, HGF/SF is enzymatically inactive [7], [25]. The α-chain contains an N-terminal finger domain (N) as well as four Kringle domains (K1–K4), and the β-chain contains a serine proteinase homology (SPH) domain [53]–[55]. HGF/SF binds to the SEMA domain of c-MET, initiating receptor dimerization, which triggers

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critical functional roles: phosphorylation of JM at Ser-975 downregulates kinase activity of c-MET; transphosphorylation of Tyr1234 and Tyr1235 of the *CR* domain enhances kinase activity; and the DS domain is a multifunctional docking site (Tyr 1349 and Tyr1356) for adaptor proteins. From a mechanistic as well as therapeutic standpoint, it is also important to fully appreciate the cross-talk between c-MET and other receptors (RON, ERBB2, Integrins, CD44, FGFR, VEGFR EGFR, and ERBB3 [13]). In HNSCC and other cancers, cross-talk between c-MET and EGFR, particularly via RAS and SRC, and between c-MET and ERBB3-EGFR (or ERBB3-ERBB2, not shown [138], [140]), are of particular relevance. Both c-MET and EGFR can activate ERBB3, initiating PI3K/AKT signaling [100], [140], [141].

phosphorylation of receptor Tyr1230, Tyr1243, and Tyr1235, leading to autophosphorylation of Tyr1349 and Tyr1356, allowing the signaling cascades described in Sect. 5.2.3 to commence (Fig. 5.1) [6], [34], [56]. As previously mentioned, in vitro studies have suggested that HGF/SF can also bind to c-MET IPT domains to initiate signaling [33]. In general, pro-HGF/SF is secreted by mesenchymal cells, and cleaved for activation, to subsequently bind to and activate c-MET on epithelial cells [57].

5.2.4 c-MET Associated Signaling

The HGF/c-MET pathway is instrumental to cellular motility, migration, and invasion, as well as to cell survival and proliferation, in both normal and cancerous cells [6], [18], [49], [58]–[63]. Once HGF/SF is bound to c-MET, tyrosine-phosphorylated c-MET then interacts directly, with phosphorylated tyrosines 1349 and 1356 acting as binding sites, with the effectors growth factor receptor-bound protein 2 (GRB2), SHC, SRC, SHP2, SHIP1, and phosphatidylinositol 3-kinase (PI3K), as well as an adapter protein, Grb2-associated binder 1 (GAB1) (Fig. 5.1) [56], [64]. GAB1 binding promotes recruitment and activation of STAT3, GRB2/SOS, PI3K, SRC, and PLCγ, and is essential for the sustained nature of c-MET initiated signaling. Downstream effectors of GRB2/SOS include the RAS/RAF/MEK/ERK mitogenic cascade and the RAC/PAK effectors that increase cell motility; PI3K activates AKT and mTOR, influencing cell survival [7], [25], [27], [65]. Cross-talk and activation of a number of additional pathways by c-MET have also been reported (Fig. 5.1; discussed in Sect. 5.4).

Downstream of the receptor, one group of cell motility/migration effectors of c-Met localize to focal adhesions, and involve proteins also responsive to integrin signaling. For example, focal adhesion kinase (p125FAK) and paxillin are downstream substrates of HGF-bound or mutationally activated c-MET [13]; c-MET activation causes reorganization of focal adhesion structure and increased cell motility/migration [66].

Another aspect of c-MET activation is initiation of the release of the cytokine interleukin 8 (IL-8, [67]). IL-8 is a proinflammatory chemokine that has been linked to angiogenic, proliferative, and survival processes in cancer. In some cases of HNSCC, IL-8 has been found to be coexpressed with HGF at high levels and this coexpression has been linked to more aggressive disease progression [16], [67], [68].

During embryogenesis, c-MET plays a crucial role by initiating the epithelial to mesenchymal transition (EMT) of myogenic progenitor cells: this process is essential for proper development of the embryo [25], [58]. Furthermore, c-MET signaling is essential for regeneration of liver and skin as well as wound healing [59], [60], [62]. Considering the normal functional roles of c-MET, the potential for aberrant, c-MET-associated proliferation, survival, and EMT-related migration leading to metastatic disease, becomes strikingly evident.

5.3 Dysregulation of HGF/c-MET in HNSCC

In many tumors, dysregulation of c-MET occurs, initiating or supporting malignant cell growth [7], [9], [10], [27], [30], [69]; furthermore, considering that c-MET is involved in EMT during embryonal development, it should not come as a surprise that c-MET has been linked to EMT in many cancers, including HNSCC. Various factors can cause dysregulation of c-MET/HGF in HNSCC, the three dominant factors being: (1) c-MET overexpression and amplification (5.3.1), (2) c-MET mutation (5.3.2), and (3) microenvironment/ligand-associated triggers (5.3.3).

5.3.1 c-MET Overexpression and Amplification

For the c-MET pathway, amplification and overexpression have been observed in multiple cancer types [6], [57], [63], [70]–[72]. Regarding HNSCC specifically, others and we have found an increase in copy number of c-MET and robust overexpression of c-MET and HGF in 60–80 % of tumors [10], [11], [16], [73]. Of particular note, c-MET expression appears to progressively increase from normal tissue, to dysplasia, to squamous cell carcinomas [11], [14], [16]. c-MET is also amplified in a subset of HNSCC tumors [11], [74], [75]. In our initial study, we observed that several cell lines had low-level c-MET copy number increases (by FISH and qPCR) and several patient-derived tumor tissues had high-level copy number increases [11]. These data have been confirmed in a recent large-scale genomic analysis, albeit c-MET amplification is only present in a small fraction of HNSCC cases (4 out of 295 tumor samples; http://www.cbioportal.org; see [76] for cBioPort information).

In addition, it has been suggested that c-MET mRNA overexpression may be important for cisplatin resistance in HNSCC [77]. Akervall et al. used cDNA microarray to evaluate the expression level of c-MET, results being subsequently verified using RT-PCR, in five chemosensitive and five chemoresistant HNSCC cell lines. Low MET expression levels were observed in the chemosensitive cell lines, compared to significantly higher expression levels in the chemoresistant cells. Similar observations were made analyzing 29 patient-derived tumor samples by immunohistochemistry (IHC). The patients had been treated with cisplatin and 5-fluorouracil and treatment responses were known: 5 of 9 (56 %) patients with tumors expressing low c-MET responded to induction chemotherapy and only 4 of 20 (20 %) patients with tumors expressing high c-MET responded. The cell line-based expression study also revealed that 30–40 other genes had expression patterns paralleling those observed for c-MET: these genes have not yet been evaluated in patient samples [10]. Further work is needed to fully elucidate the role of c-MET overexpression and amplification in this tumor type, particularly in regard to cisplatin resistance. Of note, according to the Genomics of Drug Sensitivity in Cancer Project, based on cell line sensitivity data (not HNSCC specific), c-MET amplification significantly increased sensitivity to lapatinib ($p = 0.0026$), erlotinib ($p = 0.035$), and SL0101-1 ($p = 0.0085$; http://www.cancerrxgene.org/translation/Gene/1225).

5.3.2 c-MET Mutation

A large number of genetic and protein abnormalities have been reported to be associated with the activation of c-MET in a variety of tumors [6], [7]. Furthermore, we have identified accumulation of c-MET mutations and occurrence of several single nucleotide polymorphisms (SNPs) in HNSCC [11]. Mutations in c-MET have been found to affect each of the protein's functional domains: the tyrosine kinase domain, the JM domain, the IPT domain, and the SEMA domain [11], [57], [78]–[80]. The earliest reports of germline and somatic mutations of c-MET were made for papillary renal carcinoma [81]. Thereafter, a number of gain-of-function mutations in the tyrosine kinase domain were defined in glioblastomas and in HNSCC [82]. Dulak et al. recently performed exome sequencing for 149 esophageal adenocarcinoma tumor-normal tissue pairs (whole-genome sequencing was additionally performed for 15 of these samples) and c-MET was identified to have mutations in 2 % and gain-of-function events in 6 % of tested samples: c-MET was thus considered a putative therapeutic target warranting further investigation [83]. In a phase III trial of 270 patients, Argiris et al. confirmed the previous observation that c-MET mutations are possible prognostic markers for survival and disease progression, but not for response to EGFR inhibitors [84]. The relationship between EGFR and c-MET is discussed at length in Sect. 5.4.1.

Even the most robust mutations do not abrogate the responsiveness of the c-MET receptor to HGF. We identified gain-of-function mutations or cancer-associated SNPs affecting the SEMA (HGF ligand binding) domain and the JM domain. These alterations occur in 12 % (8 of 66 analyzed tumors) of HNSCC [11], [74] and lead to hyperresponsiveness to HGF, as well as important increases in soft-agar colony formation assays for HNSCC [11]. Another interesting phenomenon we have noted is exon skipping (demonstrated in lung cancer, but not yet in HNSCC) leading to the elimination of the 47-amino acid exon encoding the JM domain, which may also alter c-MET activation [85], [86]. Cell lines, xenograft models, and tumors with mutated or elevated c-MET are particularly susceptible to c-MET inhibitors, including the small molecule c-MET inhibitor crizotinib [11], which will be further discussed in Sect. 5.5.1.2.

Finally, in GAB1, which is recruited to c-MET via direct binding (Fig. 5.1), gain-of-function mutations have been identified in some cancer settings. For example, the GAB1-T387N mutation is located in close proximity to the GRB2 binding site and appears to hamper the negative feedback potential of nonmutated T387-GAB1 [87]. Whether or not GAB1 mutations are involved in c-MET-associated resistance in HNSCC remains to be determined.

5.3.3 Microenvironment and HGF/SF

Under normal conditions, c-MET activation is regulated via tight control of HGF ligand interaction with the receptor. HGF induces upregulation of hundreds of genes,

including c-MET and the proteases needed for HGF and c-MET activation [7], [10]. The major sources of HGF are generally cells of mesenchymal origin [88]. Although the topic has been less extensively investigated than c-MET expression, it seems likely that elevated expression of HGF contributes significantly to c-MET-associated pathogenesis in HNSCC. HGF expression has been shown to be an important mechanism of c-MET activation and c-MET-associated treatment resistance in tumor types other than HNSCC, with EGFR tyrosine kinase inhibitor-resistant lung cancer being one example [89]. HGF stromal production can also induce resistance to targeted therapies (e.g., BRAF inhibition in BRAF mutant melanoma [90]); furthermore, in NSCLC, it has been observed that increased HGF accelerates the amplification of c-MET. Turke et al. speculate that increased HGF, in the presence of EGFR inhibition, creates a proliferative advantage for subclones with c-MET amplification [91]. HNSCC tumor-derived fibroblasts produce more HGF that non-tumor associated fibroblasts, and this may be one of the dominant mechanisms of c-MET activation in HNSCC [16]. HGF is also altered in 5.6 % of HNSCC cases (8 out of 302 tumor samples mutated and 9 out of 302 amplified; http://www.cbioportal.org); however, based on the preponderance of data, increased HGF protein expression levels seem to be of much greater relevance to functional effect than the mutational or amplification status of the gene.

5.4 c-MET Cross-Talk

5.4.1 c-MET and Epidermal Growth Factor (EGF) Family of Receptors

The most relevant cross-talk involving c-MET signaling is with EGFR (Her1/ERBB1), due to strong evidence suggesting c-MET as a vital part of the mechanisms enabling cancer cells to acquire resistance to EGFR-targeted drugs (discussed in detail in Sect. 5.5) [13], [14], [17], [47], [91]–[93]. A number of the c-MET effectors noted above converge with those activated by EGFR and its related effectors (Figs. 5.1) [73], [94]; it also appears, at least in NSCLC, that c-SRC is essential for EGFR to c-MET cross-talk [95]. It is the convergence of effector proteins that allows RTK cross-talk and contributes to EGFR-targeted therapy resistance in some cases [94], [96]. HGF also plays a role in regard to EGFR cross-talk, by promoting transactivation of EGFR; whereas, inhibition of EGFR has been observed to abrogate HGF-dependent phenotypes [97]. Co-immunoprecipitation of a heterodimeric MET-EGFR complex has been detected, which suggests the possibility of direct interactions; however, direct physical interaction has not been unambiguously demonstrated [13]. Garofalo et al. further connected EGFR and c-MET, when they showed that EGFR and c-MET downregulated the same miRNAs (miR-30b, miR-30c, miR221, and miR222), which, in lung cancer cells, diminished gefitinib-induced apoptosis [92].

c-MET also appears to participate in signaling cross-talk with the EGFR-related proteins HER2 (ERBB2) and HER3 (ERBB3). HER2 and HGF/c-MET signaling synergy has been demonstrated in HER2 positive breast cancer, in which HGF induced break down of cell–cell junctions, to enhance a malignant phenotype [98]. In HER2 amplified gastric cancer, activation of MET mediated active resistance to the small molecule inhibitor lapatinib [99]; these results further support the notion of c-MET/HER2 interplay.

Amplification of MET has also been reported to cause gefitinib resistance in lung cancer, by driving ERBB3 activation of PI3K. ERBB3 phosphorylation was only decreased in the simultaneous presence of a c-MET and an ERBB3 inhibitor [100]. It is also interesting to note that c-MET cannot only homodimerize to activate downstream cascades, but it can heterodimerize with ERBB3 [100], providing an additional input into cascades that report EGFR and c-MET activity. In this context, c-MET overexpression provides a source of resistance to EGFR-targeting therapies in EGFR-dependent cancers [14], [92], [96], [100]. Targeting of ERBB3/HER3 has recently become possible using inhibitors such as MM-121 [101], AV-203 [102] and ro5479599 [NCT01482377], and may be of particular interest for cases of HNSCC, as it is the primary driver of PI3K activation, one of the main pathways activated in HNSCC [94], [100] (see discussion in Chap. 6).

The definitive impact c-MET has on a variety of ERBB-expressing cancers strongly suggests similar involvement in the case of HNSCC, which frequently expresses ERBB family members [73]. Considering the meaningful, yet frequently disappointing results observed with ERBB-targeted therapeutics (response rates of 4–13 % for single agents [103], [104]), further investigation of dual- or triple-agent therapies targeting ERBB family members and HGF/c-MET simultaneously is highly desirable, as this approach effectively targets converging signaling pathways. In order to successfully develop c-MET/ERBB focused targeted therapy regiments, it will likely be important to employ genome-based medicine as described by Vogelstein et al. [105] in combination with techniques to evaluate cancer proteomics [106], to ensure identification of the patients most likely to respond to treatment, which may be a particularly successful strategy for HNSCC, in which c-MET and ERBB are both frequently expressed. Work by Xu et al. and some of our own preclinical work supports this notion and strongly suggest that targeting c-MET may enhance the efficacy of ERBB-targeted therapy in HNSCC [11], [14].

5.4.2 c-MET and SRC

Interactions between SRC and c-MET have been implicated in drug resistance and tumorigenesis in HNCs. Increased SRC kinase activity has been reported in numerous cancers, including HNSCC, and due to the known interaction of SRC, EGFR and MET (Fig. 5.1), the findings by Stabile et al. that c-SRC activation mediates resistance to erlotinib by stimulating c-MET does not come as a surprise [93]. Furthermore, c-Met activation has been demonstrated to mediate HNSCC cell survival following

SRC inhibition [107]. SRC has also been implicated with increased EMT and more aggressive tumors, a feature particularly relevant to HNSCC, a disease in which local invasion plays a dominant role and is associated with less successful locoregional control and worse outcome [3], [108]. These findings suggest that combining a SRC inhibitor, such as dasatinib, with ERBB-targeted therapy or c-MET-targeted therapy (or both) may improve the outcome for patients with erlotinib resistant HNSCC.

5.4.3 Additional Interactions

c-MET is also engaged in cross-talk with other transmembrane receptors, such as integrins, plexin B1, G-protein receptors, CD44, fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR) [13], [109]. For example, integrins play a major role in cell adherence to matrix, and multiple studies have suggested that cellular adherence is strongly associated with c-MET activity [110], [111], and may even initiate ligand-independent activation of the receptor [112]. The interaction between CD44 and c-MET is particularly curious, as CD44 can act as a co-receptor for c-MET, possibly leading to amplified HGF-MET interactions; CD44 may also be required for c-MET signaling in some instances [113]–[115]. Simultaneous inhibition of c-MET and FGFR has been observed to be superior to individual inhibition of either of the two receptors [109]. Cross-talk between VEGFR and c-MET has also been observed, suggesting an additional avenue for combination therapy [116]–[119]. All of the mentioned interactions are highly relevant and further investigations are likely to elucidate novel treatment approaches and greater understanding of the underlying biology.

5.5 HGF/c-MET-Targeted Therapy

Using siRNA, others and we have established the c-MET dependency of HNSCC tumor-derived cell lines [11], [93]. There are a large number of therapeutic strategies being utilized against HGF/c-MET (recently summarized by Sadiq et al. [9]), and a large number of inhibitors have been developed: these include multiple small molecule inhibitors (tivantinib, crizotinib; Sects. 5.5.1, 5.5.2, and 5.5.3) as well as antibodies against the receptor (MetMAb [onartuzumab], ABT700; Sect. 5.5.4) and against the ligand (ficlatuzumab, rilotumumab; Sect. 5.5.5) [9], [35]. There are currently a multitude of clinical trials evaluating c-MET or HGF targeting for several different cancers (www.clinicaltrials.gov).

The most pertinent example of potential treatment advances involving c-MET-targeted therapy is centered on reports that c-MET can be amplified specifically in the context of EGFR resistance [71]. Engleman et al. reported detection of MET amplification in 4 of 18 lung cancer specimens that had developed resistance to either gefitinib or erlotinib [100]. Turke et al. in elegant fashion showed that in

NSCLC, resistance to EGFR kinase inhibitors can be established through c-MET amplification or autocrine HGF production; furthermore, Turke et al. demonstrated that combined EGFR and c-MET inhibition was curative in vivo. Analysis of clinical data showed c-MET amplification in 4 of 27 samples from EGFR inhibitor-resistant tumor specimens [91]. Lastly, Bean et al. reported that 9 of 43 patients with acquired resistance to erlotinib or gefitinib presented with c-MET amplification; whereas among 62 untreated patients, only two presented with amplified c-MET. Interestingly, Bean et al. found no correlation between c-MET amplification and EGFR mutation status [120]. These reports strongly support the rationale of MET inhibition in specific cases; one of the obvious challenges will be to efficiently and effectively identify the proper treatment regimen for a given patient population.

Most investigations regarding c-MET and EGFR have thus far been conducted in cases of lung cancer; nevertheless, we and others have observed marked synergy of combining c-MET and EGFR inhibition in the majority of HNSCC examined [11], [14], [15]. For HNSCC, a comprehensive randomized phase II trial is ongoing [NCT016955] to evaluate the response of patients with recurrent, metastatic or inoperable HNSCC to cetuximab (Chap. 3, Fig. 3.2) with or without tivantinib (Sect. 5.5.2). One of the primary efforts, as alluded to in Sect. 5.4, is to investigate the feasibility of inhibiting c-MET as a means of overcoming resistance to other inhibitors in use for HNSCC.

5.5.1 Foretinib

Foretinib is an orally available inhibitor of MET, RON, AXL, Tie-1, KIT, PDGFR, and VEGFR. Importantly, foretinib inhibits HGF-induced MET phosphorylation and extracellular VEGF-induced kinase phosphorylation events (Fig. 5.2) [121]. In HNSCC, we have studied foretinib in a phase II single-agent clinical trial [15]. Foretinib was administered orally at 240 mg for five consecutive days of a 14-day cycle (a maximum of 30 cycles was administered). While only 14 patients were treated and no objective responses were observed, 43 % (6 of 14) of patients had tumor shrinkage of 5–21 % and the treatment was generally well tolerated [15]. The fact that foretinib was tolerable and tumor shrinkage was observed in some patients suggests initiation of larger trials, ideally combining MET and EGFR inhibitors, may be advantageous.

5.5.2 Tivantinib

More potent c-MET inhibitors than foretinib are currently being explored, namely in a randomized study of tivantinib versus tivantinib plus cetuximab [NCT01696955]. This study builds on the observed synergy of c-MET and EGFR inhibition [11], [122]. Tivantinib, a staurosporine derivative that targets dephosphorylated c-MET, has shown promising activity in several phase I and phase II trials, and is currently

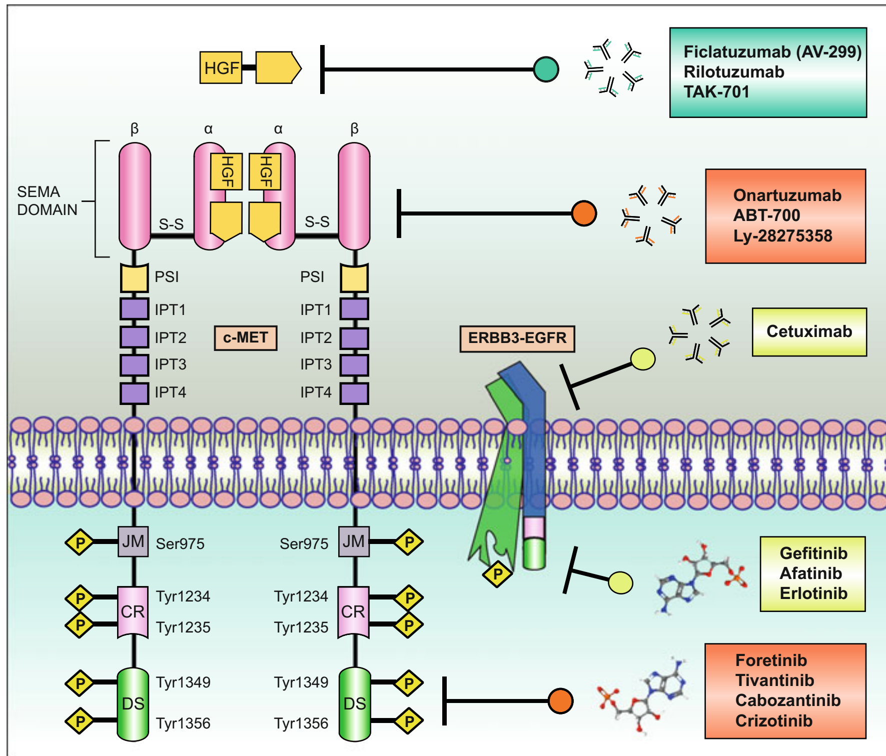

Fig. 5.2 HGF/c-MET-targeted inhibitors. Three different categories of HGF/c-MET-targeted inhibitors are currently available: HGF-targeting antibodies (*turquoise box*); c-MET-targeting antibodies (*top orange box*); and small molecules that target the intracellular domain of c-MET (*bottom orange box*). One EGFR-targeting antibody (*top yellow box*) and two small-molecule EGFR inhibitors (*bottom yellow box*; erlotinib and gefitinib) and one small-molecule EGFR/ERBB2/ERBB3 inhibitor (*bottom yellow box*; afatinib), are of particular interest for combination therapies with HGF/c-MET inhibitors (see Chap. 3 for more information on ERBB family member inhibitors).

being evaluated in a phase III trial [NCT01244191] in combination with erlotinib for the treatment of NSCLC [123], [124]. However, evidence has emerged suggesting that tivantinib has cytotoxic activity independent of interaction with c-MET, necessitating further careful evaluation of this compound [123], [125].

5.5.3 *Additional Small Molecule Inhibitors*

Cabozantinib is a promising multitargeted tyrosine kinase inhibitor (TKI) with activity against c-MET, VEGFR2, and RET that is currently being studied in a phase III clinical trial of advanced medullary thyroid cancer [NCT00704730]. Additionally, cabozantinib was assessed in combination with erlotinib in a phase Ib/II study in

patients with NSCLC: 17 % of patients achieved 30 % or greater reduction in tumor burden [126]. Crizotinib, another small molecule inhibitor, has recently emerged as a promising treatment option for anaplastic lymphoma kinase (ALK)-positive NSCLC; in addition to targeting ALK, crizotinib also inhibits c-MET [7]. Ou et al. have reported the observation that an NSCLC patient with de novo c-MET amplification but no ALK fusion protein had a sustainable response to crizotinib [127]. There are several additional small molecule c-MET inhibitors that may be worth consideration for the treatment of HNSSC [7], [126].

5.5.4 Antibodies Targeting c-MET

Onartuzumab (MetMAb) is a recombinant monoclonal antibody that binds the SEMA domain of c-MET (Fig. 5.2). By binding to c-MET, onartuzumab blocks HGF from binding and activating c-MET signaling. There is also evidence that HGF may serve as a reliable biomarker for onartuzumab target engagement [128]. Onartuzumab is currently being evaluated in phase II and phase III clinical trials: The phase III study treats patients with MET diagnostic-positive NSCLC with onartuzumab/erlotinib [NCT01456325]. Another c-MET targeting antibody currently in clinical trials [NCT01287546; NCT01602289] is LY-2875358, a humanized IgG4 monoclonal antibody. LY-2875358 binds to c-MET and thus inhibits binding of HGF, additionally, the antibody induces c-MET internalization and degradation; therefore, LY-2875358 can disrupt ligand-dependent and independent activation of c-MET [129].

5.5.5 Antibodies Targeting HGF/SF

Another therapeutic approach to inhibit HGF/c-MET signaling is by targeting HGF with antibodies (Fig. 5.2). Ficlatuzumab (AV-299) is a humanized IgG1 antibody that specifically binds HGF. In a phase II study designed to compare gefitinib as a single agent versus gefitinib and ficlatuzumab in patients with adenocarcinoma of the lung, the drug combination did not significantly improve response rate or progression-free survival [130]; however, a phase I trial with ficlatuzumab in combination with cisplatin and radiation in HNSCC is under development and may yield more encouraging results.

Rilotumumab is a fully human monoclonal antibody that also targets HGF, leading to reduced HGF/MET signaling. In a phase II study, the efficacy of rilotumumab plus mitroxantrone and prednisone was tested in castration-resistant prostate cancer. Unfortunately, the addition of rilotumumab did not have any beneficial effect [131]. On a more encouraging note, Tak-701, another humanized monoclonal antibody that targets HGF, successfully restored sensitivity to gefitinib in resistant human lung cancer cells [132]. The results with HGF-targeting antibodies strongly emphasize the necessity to carefully identify the proper patient population, i.e., the patient population that will be most responsive to treatment.

5.6 Conclusions and Future Directions

Cancer genomics have provided us with sufficient data to realize that "driver" genes can be assigned to at least 12 signaling pathways [105]. There is currently a major impetus to perform genomic evaluation of individual patients (precision medicine/oncology; [133], [134]), and to try to target multiple pathways rather than single proteins. Exploring the potential of c-MET-targeted therapy, particularly in combination with other inhibitors targeting interlinked pathways (e.g., cetuximab, erlotinib), in HNSCC may help with the transition to exclusive practice of precision medicine in oncology.

Precision medicine requires discovery and exploitation of good biomarkers. An encouraging example of biomarker based precision medicine is the use of the anti-c-MET antibody onartuzumab/MetMAb (Sect. 5.5.4) for specific cases of NSCLC: simple measurement of c-MET expression using IHC appeared to have identified a population of patients that benefited from treatment more so than patients presenting with low c-MET expressing disease [135], [136]. Spigel et al. found that patients with c-MET IHC + tumors significantly benefited, statistically and clinically, from MetMAb plus erlotinib (ME) treatment compared to placebo plus erlotinib (PE) (c-MET IHC +: overall survival of 4.6 months (PE) and 12.6 months (ME), $p=0.002$; c-MET IHC-: overall survival 9.2 months (PE) and 5.5 months (ME), $p=0.021$; [136]). Clearly, the ability to detect biomarkers, predict efficacy of available treatment options and customize treatment regimens for each individual patient is starting to emerge as a reality.

Environmental factors, though in some cases tremendously complicated to integrate into existing disease models and profiles, will also have to be better understood in order to fully implement precision medicine. For example, it currently remains unknown whether human papillomavirus virus (HPV) status impacts efficacy of HGF/c-MET-targeted agents. Preliminary evidence suggests that HGF/c-MET mutational or amplifying events are less common in HPV(+) tumors, based on early reports of the Cancer Genome Atlas Project (TCGA) [74]. Still, validation will be important, including evaluation of tumor stroma from HPV(+) HNSCC tumors.

The HGF/c-MET axis has been studied for a considerable period of time and is altered and biologically important in multiple cancer types, including in HNSCC. Early clinical data indicate that HGF/c-MET inhibition is feasible and may be beneficial in at least a subset of HNSCC patients, albeit larger clinical studies are still ongoing. However, to optimize therapeutic approaches, patient selection and identification of predictive biomarkers as well as the optimal combination with other agents will be essential. We expect, based on knowledge from other tumor types, that the most activity will occur in a subset of patients that have activated c-MET. In order to identify specific markers, tissues from patients should be obtained from ongoing c-MET HNSCC studies and analyzed for HGF/c-MET expression, mutations/SNPs, amplification and relationships with actionable downstream targets and other signaling pathways. Combinational strategies with EGFR inhibition and potentially other treatment modalities should be explored in rationally selected patient populations, in an effort to help current patients and as part of the greater effort to lay the groundwork for future treatment approaches.

References

1. Haddad RI, Shin DM. Recent advances in head and neck cancer. N Engl J Med. 2008;359(11):1143–54.
2. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer. 2011;11(1):9–22.
3. Pfister DG, et al. Head and neck cancers. J Natl Compr Cancer Netw. 2011;9(6):596–649.
4. De Herdt MJ, de Jong RJB. HGF and c-MET as potential orchestrators of invasive growth in head and neck squamous cell carcinoma. Front Bioscie-Landmark. 2008;13:2516–26.
5. Lo Muzio L, et al. Effect of c-Met expression on survival in head and neck squamous cell carcinoma. Tumor Biol. 2006;27(3):115–21.
6. Birchmeier C, et al. Met, metastasis, motility and more. Nat Rev Mol Cell Biol. 2003;4(12):915–25.
7. Blumenschein GR Jr, Mills GB, Gonzalez-Angulo AM. Targeting the hepatocyte growth factor-cMET axis in cancer therapy. J Clin Oncol. 2012;30(26):3287–96.
8. Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. Nat Rev Cancer. 2006;6(8):637–45.
9. Sadiq AA, Salgia R. Inhibition of MET receptor tyrosine kinase and its ligand hepatocyte growth factor. J Thorac Oncol. 2012;7(16 Suppl 5):S372–4.
10. Akervall J, et al. Genetic and expression profiles of squamous cell carcinoma of the head and neck correlate with cisplatin sensitivity and resistance in cell lines and patients. Clin Cancer Res. 2004;10(24):8204–13.
11. Seiwert TY, et al. The MET Receptor Tyrosine Kinase Is a Potential Novel Therapeutic Target for Head and Neck Squamous Cell Carcinoma. Cancer Res. 2009;69(7):3021–31.
12. Molinolo AA, et al. Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol. 2009;45(4–5):324–34.
13. Lai AZ, Abella JV, Park M. Crosstalk in Met receptor oncogenesis. Trends Cell Biol. 2009;19(10):542–51.
14. Xu H, et al. Dual blockade of EGFR and c-Met abrogates redundant signaling and proliferation in head and neck carcinoma cells. Clin Cancer Res. 2011;17(13):4425–38.
15. Seiwert T, et al. Phase II trial of single-agent foretinib (GSK1363089) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. Invest New Drugs. 2013;31(2):417–24.
16. Knowles LM, et al. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. Clin Cancer Res. 2009;15(11):3740–50.
17. Singleton KR, et al. A receptor tyrosine kinase network composed of fibroblast growth factor receptors, epidermal growth factor receptor, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, and hepatocyte growth factor receptor drives growth and survival of head and neck squamous carcinoma cell lines. Mol Pharmacol. 2013;83(4):882–93.
18. Park M, et al. Mechanism of met oncogene activation. Cell. 1986;45(6):895–904.
19. Cooper CS, et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature. 1984;311(5981):29–33.
20. Peschard P, Park M. From Tpr-Met to Met, tumorigenesis and tubes. Oncogene. 2007;26(9):1276–85.
21. Park M, et al. Sequence of met protooncogene Cdna has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci U S A. 1987;84(18):6379–83.
22. Bottaro DP, et al. Identification of the Hepatocyte Growth-Factor Receptor as the C-Met Protooncogene Product. Science. 1991;251(4995):802–4.
23. Rodrigues GA, Naujokas MA, Park M. Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. Mol Cell Biol. 1991;11(6):2962–70.
24. Kim ES, Salgia R. MET pathway as a therapeutic target. J Thorac Oncol. 2009;4(4):444–7.
25. Gherardi E, et al. Targeting MET in cancer: rationale and progress. Nat Rev Cancer. 2012;12(2):89–103.

26. Giordano S, et al. Tyrosine kinase receptor indistinguishable from the c-met protein. Nature. 1989;339(6220):155–6.
27. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol. 2010;11(12):834–48.
28. Gherardi E, et al. Structural basis of hepatocyte growth factor/scatter factor and MET signalling. Proc Natl Acad Sci U S A. 2006;103(11):4046–51.
29. Komada M, et al. Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin. FEBS Lett. 1993;328(1–2):25–9.
30. Chen Z. Aberrant Activation of HGF/c-MET Signaling and targeted therapy in squamous cancer, in signaling pathways in squamous cancer. In: Glick AB, Van Waes C, Editors. New York: Springer; 2011. p. 462.
31. Prat M, et al. C-terminal truncated forms of Met, the hepatocyte growth factor receptor. Mol Cell Biol. 1991;11(12):5954–62.
32. Gherardi E, et al. The sema domain. Curr Opin Struct Biol. 2004;14(6):669–78.
33. Basilico C, et al. A high affinity hepatocyte growth factor-binding site in the immunoglobulin-like region of Met. J Biol Chem. 2008;283(30):21267–77.
34. Ponzetto C, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 1994;77(2):261–71.
35. Sadiq AA, Salgia R. MET As a Possible Target for Non-Small-Cell Lung Cancer. J Clin Oncol. 2013;31(8):1089–96.
36. Ganesan R, et al. Proteolytic activation of pro-macrophage-stimulating protein by hepsin. Mol Cancer Res. 2011;9(9):1175–86.
37. Wang MH, et al. Potential therapeutics specific to c-MET/RON receptor tyrosine kinases for molecular targeting in cancer therapy. Acta Pharmacol Sin. 2010;31(9):1181–8.
38. Welm AL, et al. The macrophage-stimulating protein pathway promotes metastasis in a mouse model for breast cancer and predicts poor prognosis in humans. Proc Natl Acad Sci U S A. 2007;104(18):7570–5.
39. Yoon TM, et al. Expression of the receptor tyrosine kinase recepteur d'origine nantais and its association with tumor progression in hypopharyngeal cancer. Head Neck. 2012.
40. Yao HP, et al. The monoclonal antibody Zt/f2 targeting RON receptor tyrosine kinase as potential therapeutics against tumor growth-mediated by colon cancer cells. Mol Cancer. 2011;10.
41. Kermorgant S, Parker PJ. Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol. 2008;182(5):855–63.
42. Kermorgant S, Zicha D, Parker PJ. PKC controls HGF-dependent c-Met traffic, signalling and cell migration. EMBO J. 2004;23(19):3721–34.
43. Cho KW, et al. Identification of a pivotal endocytosis motif in c-Met and selective modulation of HGF-dependent aggressiveness of cancer using the 16-mer endocytic peptide. Oncogene. 2013;32(8):1018–29.
44. Petrelli A, et al. The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature. 2002;416(6877):187–90.
45. Li N, et al. Specific Grb2-mediated interactions regulate clathrin-dependent endocytosis of the cMet-tyrosine kinase. J Biol Chem. 2007;282(23):16764–75.
46. Singleton PA, et al. CD44 regulates hepatocyte growth factor-mediated vascular integrity—role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin. J Biol Chem. 2007;282(42):30643–57.
47. Ogi S, et al. Sorting nexin 2-mediated membrane trafficking of c-Met contributes to sensitivity of molecular-targeted drugs. Cancer Sci. 2013;104(5):573–83.
48. Kamei T, et al. Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells—regulation by Rho, Rac and Rab small G proteins. Oncogene. 1999;18(48):6776–84.
49. Joffre C, et al. A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol. 2011;13(7):827–U227.
50. Kermorgant S, Parker PJ. c-met signalling—spatio-temporal decisions. Cell Cycle. 2005;4(3):352–5.

51. Ultsch M, et al. Crystal structure of the NK1 fragment of human hepatocyte growth factor at 2.0 A resolution. Structure. 1998;6(11):1383–93.
52. Donate LE, et al. Molecular Evolution and Domain-Structure of Plasminogen-Related Growth-Factors (Hgf/Sf and Hgf1/Msp). Protein Sci. 1994;3(12):2378–94.
53. Kirchhofer D, et al. Structural and functional basis of the serine protease-like hepatocyte growth factor beta-chain in Met binding and signaling. J Biol Chem. 2004;279(38):39915–24.
54. Stamos J, et al. Crystal structure of the HGF beta-chain in complex with the Sema domain of the Met receptor. EMBO J. 2004;23(12):2325–35.
55. Chirgadze DY, et al. Crystal structure of the NK1 fragment of HGF/SF suggests a novel mode for growth factor dimerization and receptor binding. Nat Struct Biol. 1999;6(1):72–9.
56. Xu Y, et al. Receptor-type protein tyrosine phosphatase beta (RPTP-beta) directly dephosphorylates and regulates hepatocyte growth factor receptor (HGFR/Met) function. J Biol Chem. 2011;286(18):15980–8.
57. Sattler M, et al. The role of the c-Met pathway in lung cancer and the potential for targeted therapy. Ther Adv Med Oncol. 2011;3(4):171–84.
58. Bladt F, et al. Essential role for the C-Met receptor in the migration of myogenic precursor cells into the limb bud. Nature. 1995;376(6543):768–71.
59. Borowiak M, et al. Met provides essential signals for liver regeneration. Proc Natl Acad Sci U S A. 2004;101(29):10608–13.
60. Chmielowiec J, et al. c-Met is essential for wound healing in the skin. J Cell Biol. 2007;177(1):151–62.
61. Huang FI, et al. Hepatocyte growth factor activates Wnt pathway by transcriptional activation of LEF1 to facilitate tumor invasion. Carcinogenesis. 2012;33(6):1142–8.
62. Huh CG, et al. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A. 2004;101(13):4477–82.
63. Kim CH, et al. c-MET expression as an indicator of survival outcome in patients with oral tongue carcinoma. Head neck-J Sci Spec. 2010;32(12):1655–64.
64. Weidner KM, et al. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature. 1996;384(6605):173–6.
65. Comoglio PM, Giordano S, Trusolino L. Drug development of MET inhibitors: targeting oncogene addiction and expedience. Nat Rev Drug Discov. 2008;7(6):504–16.
66. Grotegut S, et al. Hepatocyte growth factor induces cell scattering through MAPK/Egr-1-mediated upregulation of Snail. EMBO J. 2006;25(15):3534–45.
67. Dong G, et al. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. Cancer Res. 2001;61(15):5911–8.
68. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res. 2008;14(21):6735–41.
69. Cecchi F, Rabe DC, Bottaro DP. Targeting the HGF/Met signalling pathway in cancer. Eur J Cancer. 2010;46(7):1260–70.
70. Miyamoto M, et al. Prognostic significance of overexpression of c-Met oncoprotein in cholangiocarcinoma. Br J Cancer. 2011;105(1):131–8.
71. Sierra J. c-MET as a potential therapeutic target and biomarker in cancer. Ther Adv Med Oncol. 2011;3(S1):S21–35.
72. Ma PC, et al. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev. 2003;22(4):309–25.
73. Mehra R, et al. Protein-intrinsic and signaling network-based sources of resistance to EGFR- and ERBB family-targeted therapies in head and neck cancer. Drug Resist Updat. 2011;14(6):260–79.
74. Hayes DN, Grandis J, El-Naggar AK. Comprehensive genomic characterization of squamous cell carcinoma of the head and neck in the Cancer Genome Atlas in AACR Annual Meeting 2013, AACR: Washington, DC.

75. Bandla S, et al. Comparative genomics of esophageal adenocarcinoma and squamous cell carcinoma. Ann Thorac Surg. 2012;93(4):1101–6.
76. Gao JJ, et al. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Sci Signal. 2013;6(269).
77. Akervall J, et al. Genetic and expression profiles of squamous cell carcinoma of the head and neck correlate with cisplatin sensitivity and resistance in cell lines and patients. Clin Cancer Res. 2004;10(24):8204–13.
78. Tyner JW, et al. MET receptor sequence variants R970C and T992I lack transforming capacity. Cancer Res. 2010;70(15):6233–7.
79. Tengs T, et al. A transforming MET mutation discovered in non-small cell lung cancer using microarray-based resequencing. Cancer Lett. 2006;239(2):227–33.
80. Lengyel E, Sawada K, Salgia R. Tyrosine kinase mutations in human cancer. Curr Mol Med. 2007;7(1):77–84.
81. Schmidt L, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet. 1997;16(1):68–73.
82. Di Renzo MF, et al. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. Oncogene. 2000;19(12):1547–55.
83. Dulak AM, et al. Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. Nat Genet. 2013;45(5):478–U37.
84. Argiris A, et al. Phase III randomized, placebo-controlled trial of docetaxel with or without gefitinib in recurrent or metastatic head and neck cancer: an eastern cooperative oncology group trial. J Clin Oncol. 2013;31(11):1405–14.
85. Ma PC, et al. c-MET mutational analysis in small cell lung cancer: Novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res. 2003;63(19):6272–81.
86. Ma PC, et al. Functional expression and mutations of c-met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 2005;65(4):1479–88.
87. Ortiz-Padilla C, et al. Functional characterization of cancer-associated Gab1 mutations. Oncogene. 2013;32(21):2696–702.
88. Sonnenberg E, et al. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J Cell Biol. 1993;123(1):223–35.
89. Takeuchi S, et al. Dual inhibition of met kinase and angiogenesis to overcome HGF-induced EGFR-TKI resistance in EGFR mutant lung cancer. Am J Pathol. 2012;181(3):1034–43.
90. Straussman R, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012;487(7408):500–U118.
91. Turke AB, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell. 2010;17(1):77–88.
92. Garofalo M, et al. EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers. Nat Med. 2012;18(1):74–82.
93. Stabile LP, et al. c-Src activation mediates erlotinib resistance in head and neck cancer by stimulating c-Met. Clin Cancer Res. 2013;19(2):380–92.
94. Guo A, et al. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci U S A. 2008;105(2):692–7.
95. Dulak AM, et al. HGF-independent potentiation of EGFR action by c-Met. Oncogene. 2011;30(33):3625–35.
96. Tang Z, et al. Dual MET-EGFR combinatorial inhibition against T790M-EGFR-mediated erlotinib-resistant lung cancer. Br J Cancer. 2008;99(6):911–22.
97. Bonine-Summers AR, et al. Epidermal growth factor receptor plays a significant role in hepatocyte growth factor mediated biological responses in mammary epithelial cells. Cancer Biol Ther. 2007;6(4):561–70.
98. Khoury H, et al. HGF converts ERBB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell. 2005;16(2):550–61.

99. Chen CT, et al. MET activation mediates resistance to lapatinib inhibition of HER2-amplified gastric cancer cells. Mol Cancer Ther. 2012;11(3):660–9.
100. Engelman JA, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316(5827):1039–43.
101. Schoeberl B, et al. An ERBB3 antibody, MM-121, is active in cancers with ligand-dependent activation. Cancer Res. 2010;70(6):2485–94.
102. Meetze K, et al. The anti-tumor activity of the ERBB3 inhibitory antibody AV-203 in patient derived tumor explant models. Eur J Cancer. 2012;48:126.
103. Vermorken JB, et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol. 2007;25(16):2171–7.
104. Soulieres D, et al. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. J Clin Oncol. 2004;22(1):77–85.
105. Vogelstein B, et al. Cancer genome landscapes. Science. 2013;339(6127):1546–58.
106. Brennan DJ, et al. Antibody-based proteomics: fast-tracking molecular diagnostics in oncology. Nat Rev Cancer. 2010;10(9):605–17.
107. Sen B, et al. Distinct interactions between c-Src and c-Met in mediating resistance to c-Src inhibition in head and neck cancer. Clin Cancer Res. 2011;17(3):514–24.
108. Mandal M, et al. Epithelial to mesenchymal transition in head and neck squamous carcinoma: association of Src activation with E-cadherin down-regulation, vimentin expression, and aggressive tumor features. Cancer. 2008;112(9):2088–100.
109. Singleton KR, et al. A receptor tyrosine kinase network composed of fibroblast growth factor receptors, epidermal growth factor receptor, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, and hepatocyte growth factor receptor drives growth and survival of head and neck squamous carcinoma cell lines. Mol Pharmacol. 2013;83(4):882–93.
110. Nakamura Y, et al. Constitutive activation of c-Met is correlated with c-Met overexpression and dependent on cell-matrix adhesion in lung adenocarcinoma cell lines. Cancer Sci. 2008;99(1):14–22.
111. Chen SY, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol Cell Biol. 2006;26(13):5155–67.
112. Wang R, Kobayashi R, Bishop JM. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. Proc Natl Acad Sci U S A. 1996;93(16):8425–30.
113. Orian-Rousseau W, et al. Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-Actin. Mol Biol Cell. 2007;18(1):76–83.
114. Matzke A, et al. Haploinsufficiency of c-met in cd44(-/-) mice identifies a collaboration of CD44 and c-met in vivo. Mol Cell Biol. 2007;27(24):8797–806.
115. Orian-Rousseau V, et al. CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev. 2002;16(23):3074–86.
116. Lin YM, et al. Hepatocyte growth factor increases vascular endothelial growth factor-A production in human synovial fibroblasts through c-Met receptor pathway. PLoS One. 2012;7(11):e50924.
117. Eder JP, et al. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res. 2009;15(7):2207–14.
118. Sulpice E, et al. Cross-talk between the VEGF-A and HGF signalling pathways in endothelial cells. Biol Cell. 2009;101(9):525–39.
119. Xin XH, et al. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. Am J Pathol. 2001;158(3):1111–20.
120. Bean J, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A. 2007;104(52):20932–7.

121. Dufies M, et al. Mechanism of action of the multikinase inhibitor Foretinib. Cell Cycle. 2011;10(23):4138–48.
122. Xu L, et al. Combined EGFR/MET or EGFR/HSP90 Inhibition Is Effective in the Treatment of Lung Cancers Codriven by Mutant EGFR Containing T790M and MET. Cancer Res. 2012;72(13):3302–11.
123. Michieli P, Di Nicolantonio F. Targeted therapies: tivantinib-a cytotoxic drug in MET inhibitor's clothes? Nat Rev Clin Oncol. 2013.
124. Scagliotti GV, et al. Rationale and design of MARQUEE: a phase III, randomized, double-blind study of tivantinib plus erlotinib versus placebo plus erlotinib in previously treated patients with locally advanced or metastatic, nonsquamous, non-small-cell lung cancer. Clin Lung Cancer. 2012;13(5):391–5.
125. Basilico C, et al. Tivantinib (ARQ197) Displays Cytotoxic Activity That Is Independent of Its Ability to Bind MET. Clin Cancer Res. 2013;19(9):2381–92.
126. Scagliotti GV, Novello S, von Pawel J. The emerging role of MET/HGF inhibitors in oncology. Cancer Treat Rev. 2013.
127. Ou SH, et al. Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. J Thorac Oncol. 2011;6(5):942–6.
128. Penuel J, Smith JC, Shen SQ. Integer programming models and algorithms for the graph decontamination problem with mobile agents. Networks. 2013;61(1):1–19.
129. Zeng W, et al. Abstract 2734: c-Met antibody LY2875358 (LA480) shows differential antitumor effects in non-small cell lung cancer. Cancer Res. 2012;72(8).
130. D'Arcangelo M, Cappuzzo F. Focus on the potential role of ficlatuzumab in the treatment of non-small cell lung cancer. Biologics. 2013;7:61–8.
131. Ryan CJ, et al. Targeted MET inhibition in castration-resistant prostate cancer: a randomized phase II study and biomarker analysis with rilotumumab plus mitoxantrone and prednisone. Clin Cancer Res. 2013;19(1):215–24.
132. Okamoto W, et al. TAK-701, a humanized monoclonal antibody to hepatocyte growth factor, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. Mol Cancer Ther. 2010;9(10):2785–92.
133. Mirnezami R, Nicholson J, Darzi A. Preparing for precision medicine. N Engl J Med. 2012;366(6):489–91.
134. Garraway LA, Verweij J, Ballman KV. Precision oncology: an overview. J Clin Oncol. 2013;31(15):1803–5.
135. Spigel DR, et al. Treatment rationale study design for the MetLung trial: a randomized, double-blind phase III study of Onartuzumab (MetMAb) in combination with erlotinib versus erlotinib alone in patients who have received standard chemotherapy for stage IIIB or IV met-positive non-small-Cell Lung Cancer. Clin Lung Cancer. 2012;13(6):500–4.
136. Spigel D, et al. Final efficacy results from OAM4558 g, a randomized phase II study evaluating MetMAb or placebo in combination with erlotinib in advanced NSCLC. J Clin Oncol. 2011;29(15 (Supplemental; abstr 7505)).
137. Xie Q, et al. Hepatocyte growth factor (HGF) autocrine activation predicts sensitivity to MET inhibition in glioblastoma. Proc Natl Acad Sci U S A. 2012;109(2):570–5.
138. Karamouzis MV, Konstantinopoulos PA, Papavassiliou AG. Targeting MET as a strategy to overcome crosstalk-related resistance to EGFR inhibitors. Lancet Oncology. 2009;10(7): 709–17.
139. Stamos J, et al. Crystal structure of the HGF beta-chain in complex with the Sema domain of the met receptor. EMBO J. 2004;23(12):2325–35.
140. Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer. 2009;9(7):463–75.
141. Yamamoto H, Toyooka S, Mitsudomi T. Impact of EGFR mutation analysis in non-small cell lung cancer. Lung Cancer. 2009;63(3):315–21.

Chapter 6
Insulin-like Growth Factor-1 Receptors in Head and Neck Cancer

Steven A. Rosenzweig and Casey O. Holmes

Abstract The insulin-like growth factor 1 receptor (IGF-1R) has emerged as a significant contributor to the progression and metastatic spread of a number of cancers, including head and neck cancer (HNC). The receptor is most frequently overexpressed in cancer cells, with no evidence of activating mutations to date. Because this receptor exhibits robust activation of PI3K/Akt leading to mTOR activation and anti-apoptotic signaling, it has the capability of enhancing the tumorigenic signaling of other growth factors and cytokines. It is this property of the IGF-1R that has led to its becoming a viable target for cancer therapeutic strategies. In HNC, the IGF-1R has been shown to be involved in the activation of multiple signaling pathways and to be responsible, in part, for some cases of acquired resistance to chemotherapeutics. In this chapter, the role of the IGF-1R and the components of this system will be discussed and their roles in HNC will be evaluated.

Keywords Insulin-like growth factor 1 receptor · Insulin Receptor Substrate 1 and 2 · Invadopodia · Biomarker · Metastasis

Abbreviations

COX-2	Cyclooxygenase-2
CCL5	C-C motif ligand 5
CCR5	C-C motif receptor 5
DOCK3	Dedicator of cytokinesis 3
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EP2/EP4	Prostaglandin E2/E4 receptor

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Erk	Extracellular signal-regulated kinase
FFPE	Formalin-fixed, paraffin-embedded
GEF	Guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
HB-EGF	Heparin-binding-epidermal growth factor
HIF	Hypoxia-inducible factor
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HuR	Human antigen R
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP	Insulin-like growth factor binding protein
IRS	Insulin receptor substrate
IR	Insulin receptor
kDa	kilodaltons
M6P	Mannose-6-phosphate
MAPK	Mitogen-activated protein kinase
miR	microRNA
MMP	Matrix metalloproteinase
mTORC1	mammalian target of rapamycin complex 1
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9
PAK1	p21 activated kinase 1
PI 3K	PhosphatidylinositoI 3-kinase
Ptdins-4,5 P2	PhosphatidylinositoI-4,5 bisphosphate
Ptdins-3,4,5 P3	PhosphatidylinositoI-3,4,5 trisphosphate
PDK-1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology
PTB	Phosphotyrosine binding
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated, coiled-coil containing protein kinase
RPE	Retinal pigment epithelium
RTK	Receptor tyrosine kinase
RTKI	Receptor tyrosine kinase inhibitor
SH2	Src homology 2
Shc	Src homology and collagen containing
S1PR	Sphingosine-1-phosphate receptor
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor

Head and neck squamous cell carcinoma (HNSCC) accounts for more than 90 % of all pharyngeal and oral cavity tumors. With nearly 8,000 deaths a year nationally, it constitutes approximately 4 % of all cancers in the USA and is one of the six most frequent cancers worldwide. Tobacco and alcohol use are the primary risk

factors in this disease, however, additional factors contribute to this cancer [13]. Consequently, novel molecular markers for HNSCC are being sought as potential new therapeutic targets. In that context, overexpression of some highly validated proteins, such as epidermal growth factor receptor (EGFR), have been reported in a variety of human cancers, including breast, lung, colon, prostate, esophageal, brain, ovary, and head and neck cancer (HNC, [19], [33], [79]), and therapeutics targeting the EGFR have proved valuable in the clinic [29], [102], as discussed in Chap. 4. Similarly, dysregulation of several components of the insulin-like growth factor (IGF) pathway have been observed in a variety of cancers including HNC [95]. Overexpression of the insulin-like growth factor 1 receptor (IGF-1R) as well as other components of the IGF pathway have been identified both in human tissue samples and cell lines by our lab and others [4], [28], [89]. Additionally, preclinical as well as clinical studies have shown that IGF-1R synergizes with EGFR to produce a malignant phenotype [80], [85]. These findings support a role for targeting both the IGF-1R and EGFR for optimal cancer treatment.

The role of IGF-1R as a cancer promoter hinges on its ability to signal through both the ERK/MAPK pathway and the AKT pathway leading to cell cycle progression, growth, survival, and differentiation [95]. Several novel inhibitory molecules have been developed to target the IGF pathway including IGF-1R monoclonal antibodies such as figitumumab and cixutumumab, small molecule tyrosine kinase inhibitors such as BMS-754807 and OSI-906, neutralizing antibodies to IGFs such as MEDI-573 and BI-836845, and recombinant IGF binding proteins [95], [106]. The role of IGF-1R in HNC progression, its cross talk with other pathways, and therapeutic targeting of this pathway will be discussed herein.

6.1 IGF-1R in Normal Cellular Function

The IGF system (Fig. 6.1) consists of three receptors: IGF-1R, IGF-2R, and the insulin receptor (IR) and three ligands: IGF-1, IGF-2, and insulin. In addition six IGF-binding proteins (IGFBP-1-6) are secreted by many cell types and serve to attenuate the actions of IGF ligands. Among these, the IGF-1R and IR are most relevant to the enhancement of HNC. These type 1 receptor tyrosine kinases (RTKs) are initially synthesized as single-chain precursors that are disulfide bonded and cleaved within the endoplasmic reticulum and Golgi complex to first yield a heterodimer and finally a heterotetramer. Once the receptor has reached the cell surface, IGF-1, IGF-2 or insulin binding to IGF-1R induces a conformational change resulting in receptor transphosphorylation [20], [101]. This results in the immediate recruitment of, docking to, and tyrosine phosphorylation of insulin receptor substrate (IRS)-1 (or IRS-2) [8]. The IRS proteins are the immediate downstream effectors of the IGF-1R, serving as scaffold proteins responsible for docking effector proteins. The IR signaling pathway, like the IGF-1R pathway, signals through IRS proteins. The differences in the roles of IR and IGF-1R are primarily based on tissues in which each receptor type is expressed [68]. The growth-promoting effects of IGF-1, IGF-2 and insulin are believed to be regulated by IGF-1R signaling, while the IR is responsible

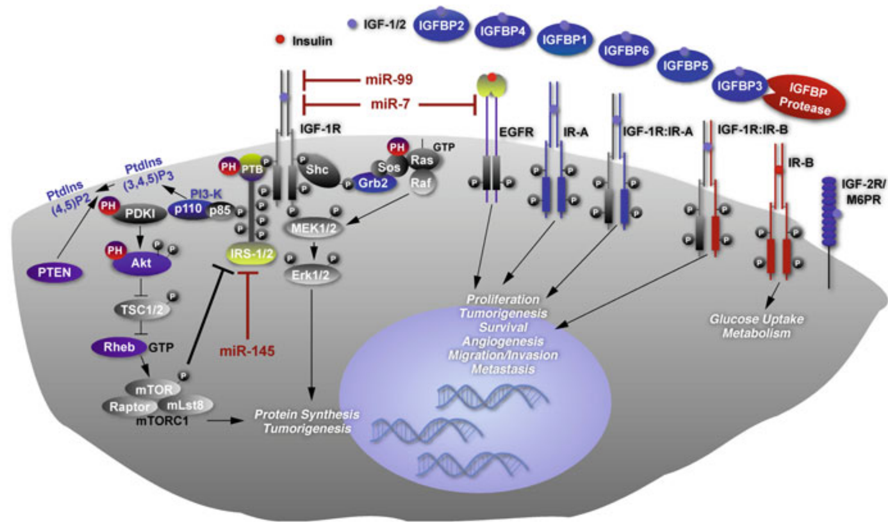

Fig. 6.1 *IGF-1* receptor signaling and regulation. *IGF-1Rs* action is negatively regulated by *IGF-BPs,* which are, in turn, susceptible to protease action. In addition, IGF-1R expression is influenced by *miR-7,* while *IRS-1, IRS-2,* and additional signaling effectors are regulated by *miR-145*

for the metabolic actions of insulin. Consistent with this, IGF-2 and IR-A (see below) are predominantly expressed in fetal tissues to aid growth-promoting actions. This is also true for cancer tissues, where the existence of hybrid heteroteramers may also affect signaling.

It has been postulated that IRS-1 signals to components of growth promoting and anti-apoptotic pathways and its expression is particularly important for cell transformation [8]. IRS-1 contains 18 potential sites of tyrosine phosphorylation that may serve as Src homology-2 (SH2) domain or phosphotyrosine-binding (PTB) domain-binding sites for docking downstream effectors. The best documented IGF-1R/IRS-1 effector pathway involves activation of Akt, which leads to stimulation of mTORC1 and proliferative, prosurvival anti-apoptotic signaling that contributes to the tumorigenic process. This pathway is initiated following the binding of phosphorylated IRS-1 to an SH2 domain of the p85 regulatory subunit of PI-3 Kinase (PI-3K) [52]. The catalytic p110 subunit then moves to the membrane to phosphorylate phosphatidylinositol-4,5 bisphosphate (Ptdins-4,5 P2) in the three position to form Ptdins-3,4,5 P3. Ptdins-3,4,5 P3 serves as a binding site for proteins containing pleckstrin homology (PH) domains, such as Akt, many of which are targets of phosphoinositide-dependent kinases 1 and 2 (PDK 1 and 2 [83]). This differs from other RTKs, which, following transphosphorylation, directly bind to and activate immediate downstream effectors such as PI-3K. IGF-1R can also use this direct signaling mechanism via the extracellular signal regulated kinases (ERKs 1/2) where the signaling protein Src homologous and collagen containing (Shc)-66 binds directly to the phosphorylated IGF-1R after IGF-1 stimulation [91] or via direct linkage to PDK1 which has recently been shown to provide procancer signaling critical for IGF-1-mediated survival [2].

The six soluble IGFBPs primarily act as IGF-1/2 “antagonists” that bind their ligands with high affinity and block their access to the IGF-1R [77]. In certain cases however, preincubation of cells with IGFBP-3 before IGF-1 treatment leads to the accumulation of cell-bound forms of IGFBP-3 with lowered affinity for IGF, which may instead, enhance the IGF-1/IGF-1R interaction. However, this mechanism has never been proven explicitly [26].

Beyond the IGF-1R, the IR also impacts cell growth signaling in normal cells and in cancer. Two isoforms of the human IR exist, IR-A and IR-B, resulting from alternative splicing of the 22 exon IR gene [9]. IR-A is truncated by 12 amino acids relative to IR-B, due to skipping of exon 11, which encodes residues 717–728 at the C-terminal end of the α-subunit. IR-A is widely distributed across most tissues, whereas IR-B is present in liver, muscle, adipose, and kidney and regulates metabolism and glucose uptake [9]. Of note, IR-A is expressed in fetal tissues and in cancer cells; it preferentially binds IGF-2 and regulates growth-promoting actions [27], [81]. Hybrid receptors consisting of IGF-1R:IR-A or IGF-1R:IR-B hybrid heterotetramers bind to IGF-2 or insulin and IGF-1, respectively, and participate in cancer cell signaling [66] (Fig. 5.1).

6.2 The IGF-1R and Cancer

Baserga and coworkers first showed that IGF-I signaling was an absolute requirement for viral transformation of cells by transfecting mouse embryonic fibroblasts that were either IGF1-R null or wild type with a *Simian virus 40* (SV40) plasmid and assaying for cell growth. IGF-1R null cells were resistant to transformation, but could be prompted to transform if they were first transfected with an IGF-1R plasmid [82]. It was subsequently shown that many oncogenes, including v-Src and the heterotrimeric G protein Galpha13, require the IGF-1R signaling pathway, but not necessarily the receptor itself, to exhibit transforming activity [7], [8], [69] This requirement is consistent with the prosurvival signaling properties of IGF-1R mediated by Akt [94]. More recently, a complex between the IGF-1R (or IR) with polyoma virus middle T (PyVmT) antigen was reported [65]. Of note, ligand binding to receptor increases the formation of these complexes, their tyrosine phosphorylation, and recruitment of c-Src and phospholipase-$C_{\gamma 1}$, leading to PyVmT activation and tumor initiation [65].

Beyond this, growth-promoting effects of the IGF-1R include its role in influencing the microenvironment to enhance tumorigenesis. For example, the IGF-1R has been associated with creating a permissive environment for liver metastasis by increasing autocrine IL-6 signaling and decreasing apoptosis in a STAT-dependent manner [53]. This creation of a permissive function supports activity of other oncogenic drivers mutated in cancers with IGF-1R involvement.

The requirement for functional IGF-1R expression in supporting cell transformation underscores the importance of autocrine and paracrine IGF-2 and IGF-1 action in tumors and the tumor microenvironment, respectively, in supporting tumorigenic

progression. The autocrine and paracrine functions of the two principal ligands for IGF-1R, IGF-1, and IGF-2, have been reported to be dysregulated in many cancers. Recent work to determine how the IGF pathway may play a role in cross talk between tumor and stroma in HNC has shown that coculture stimulation of keratinocytes with squamous cell carcinoma fibroblasts causes changes in fibroblast expression of keratin 19 and vimentin that are due, in part, to actions by IGF-2 [93]. This indicates that the IGF pathway acts not only through enhanced proliferation of cells, but also by promoting epithelial to mesenchymal transition (EMT) via epithelial and stromal cell cross talk using IGF-2. In normal cells, IGF-2 is imprinted and only expressed from the paternal allele, limiting its expression; loss of imprinting results in IGF-2 overexpression [21]. The IGF-2 gene is the most overexpressed gene in colorectal cancer [108], consistent with signaling by this ligand enhancing tumorigenesis [16], [69], [82]. IGF-1 upregulation has also been reported in a variety of cancers, including HNC [89], [105].

Epidemiologic studies have shown that high circulating levels of IGF-1 are associated with increased risk of second primary tumors (SPTs) of the head and neck [104] as well as breast [34], prostate [14], lung [107], and colon [60]. Autocrine and paracrine IGF signaling is also regulated by the IGFBPs in colonic myofibroblasts. In normal tissues, IGFBPs bind and sequester IGFs, limiting their biological accessibility. In cancer, upregulated matrix metalloproteinase-7 (MMP7)-mediated cleavage of IGFBP-5 releases bound IGF-2 which can then act as a myofibroblast mitogen [37]. Related to this, methylation of the IGFBP-3 promoter, resulting in decreased local IGFBP-3 expression and hence more bioavailable IGF, has been associated with poor prognosis in non-small cell lung cancer (NSCLC) [15]. IGF-1R signaling leads to cell transformation [5], [6], enhanced tumorigenesis, and a switch from antiapoptotic to invasive/metastatic signaling in cancer [56].

In cancer, the IGF-1R pathway engages in cross talk with other critical oncogenic signaling proteins. For example, elevated expression of IGF-1 activates EGFR by stimulating a cell surface matrix metalloproteinase (MMP), which in turn cleaves the membrane-tethered ligand heparin-binding EGF (HB-EGF), enabling it to bind to and activate the EGFR [77]. Recent studies on IGF-1R and EGFR signal cross talk revealed that PDK1 is tyrosine phosphorylated by and binds directly to the IGF-1R, providing an alternative mode of receptor cross talk. In addition to IGF-1R crosstalk to the EGFR, EGFR activation can also enhance IGF-1R signaling by mediators of IGF-1R action, such as IRS-1 [11]. Cross talk between IGF-1 and vascular endothelial growth factor (VEGF) signaling has also been described. IGF-1 treatment of retinal pigment epithelium (RPE) increases VEGF expression independent of hypoxia [72], with IGF-1R positively regulating ERK1/2 production [90]. Other work supporting interaction of these pathways indicates that IGF-1 collaborates with VEGF to support vascularization of the retina in infant development [36]. We have investigated the role of IGF-1/VEGF cross talk in malignancy. We found that IGF1 enhances VEGF expression both by increasing expression of hypoxia inducible factor-1 α (HIF-1α), and through a HIF-1α independent role [87]. Both the ERK1/2 and PI3K/Akt pathways were shown to be central to IGF regulation of VEGF production; PI3K/Akt through promoter activation and secretion of VEGF, and ERK1/2 by promoting S phase entry, a critical step in cell cycle progression [88].

IGF-1R also is engaged in cross signaling with the estrogen and progesterone receptors [92], [97]. Breast cancer cell resistance to estrogen deprivation leads to alternative signaling pathways and the association of estrogen receptor-α with Shc, Src, EGFR, and the IGF-1R [91]. IGF-1R/EGFR cross talk has been described in normal human mammary epithelial cells [1], and in tamoxifen-resistant MCF-7 cells where increased sensitivity to the proliferative effects of IGF-1/2 following estradiol or tamoxifen treatment was blocked by treatment with the c-Src inhibitor SU6656 or the anti-IGF-1R monoclonal antibody AG1024 [49]. IGF-1R signaling was also shown to activate the sphingosine-1-phosphate receptor (S1PR), a GPCR that regulates cell proliferation and migration [24]. Another GPCR activated by the IGF-1R is the (C-C motif) receptor 5 (CCR5), by inducing expression of the CCR5 ligand, chemokine (C-C motif) ligand 5 (CCL5, also referred to as RANTES) [63]. COX2 activation then increases prostaglandin E2 (PGE2) production from arachidonic acid. This allows PGE2 to act on EP2 and EP4 receptors to elevate cAMP levels, in turn resulting in the expression of amphiregulin, an EGFR ligand. These events further demonstrate the concept that IGF-1R signaling synergizes with other RTKs and GPCRs to modulate cell proliferation, tumorigenesis, and metastasis.

6.3 Role of the IGF-1R in HNC

While less studied as a therapeutic target in HNC compared to the EGFR, compelling evidence exists for a role of the IGF/IGF-IR system in human neoplasia, which has led to inhibition of IGF-1R-signaling as an attractive strategy for treating cancers such as HNC [76]. IGF1-Rs are overexpressed in the majority of HNC cell lines tested [89]. Primary human tumor specimens overexpress IGF-1Rs, IGF-1, and IGF-2; they also show reduced levels of IGFBP-2, increasing IGF-1/2 access to the IGF-1R [89]. Similarly, low IGFBP-3 concentrations are associated with increased risk for tongue cancer and that IGFBP-3 levels may serve as a prognostic marker for aggressive HNC [67].

The mechanism of oncogenic promotion by the IGF system hinges on its ability to stimulate proliferation and migration, and on its critical role in oncogene transformation [70] as well as in its ability to cross talk with other receptors and stimulate angiogenesis. One role of IGF-1R activation is to induce EGFR transactivation, or EGFR kinase activity, for ERK activation [50]. This is mediated indirectly via receptor-activated shedding of EGFR ligands including HB-EGF [53] and amphiregulin [109] (see above) or directly through IGF-1R/EGFR complex formation [50]. In HNC cell lines, resistance to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib was shown to be the result of increased IGF-1R activity, suggesting that coinhibition of both the EGF and IGF-1 receptors may provide a more effective targeting in this cancer [42].

Another role of IGF-1R signaling in HNC is to increase VEGF-A expression and the autocrine/paracrine activation of VEGFR2 [39], [88]. Recent work has suggested a novel role for IGF-1R regulation of VEGF, distinct from its role in mediating angiogenesis, in which VEGF enhances signaling pathways promoting the invasive

behavior of HNSCC cells [3]. $VEGF_{165}$, an isoform of VEGF-A, is the principal isoform detected and the one most commonly overexpressed in solid tumors. Using a phosphotyrosine proteomics analysis on the HNC SCC-9 cell line, we identified a cluster of proteins involved in focal adhesions, cell motility and invasion including focal adhesion kinase (FAK), paxillin, cortactin and neural precursor cell expressed developmentally downregulated 9 (NEDD9/HEF1 [96]) as induced by VEGF [58]. NEDD9 expression was required for VEGF-induced cell migration and invasion and the formation of invadopodia; organelles responsible for cancer-cell invasion. Significantly, NEDD9 expression has been linked to the metastatic signatures of glioblastoma [64] and melanoma [48]. Immunohistochemical and deep sequencing analysis of human HNC specimens revealed that NEDD9 expression was highest in the most advanced tumors and those tumors expressing high NEDD9 levels had the greatest chance for metastasis [57]. The specific signaling pathway(s) coordinated by NEDD9 remains a fertile area of research. VEGF and other growth factors emanating from tumor cells and surrounding stroma may also influence this signaling paradigm. This pathway was identified in multiple HNC cell lines, indicating that VEGF regulates HNC cell migration, invasion, MMP expression, and invadopodia formation in an IGF-1R/NEDD9-dependent manner [31], [58].

6.4 IGF and IGFBP Proteins as Prognostic Biomarkers in HNC

Although the mechanism of cancer promotion via the IGF-1R remains incompletely delineated, over the years several studies have shown that dysregulation of the IGF pathway consistently occurs in HNC [28], [41], [98]. A considerable amount of effort has gone into determining whether serum levels of IGF system components, notably IGF-1 and IGFBP-3, might serve as prognostic biomarkers in breast, prostate, colon, and other cancers, with mixed results [71]. The outcomes of these studies have undergone significant reevaluation and meta-analyses with the results being inconclusive. Nonetheless, each cancer requires thorough evaluation, as in the case of breast cancer where circulating IGF-1 was positively associated with breast-cancer risk for estrogen-receptor-positive tumors [25]. A recent prospective analysis of IGF-1, IGF-2, IGFBP-3, and IGFBP-2 serum levels at the start and end of radiotherapy evaluated 17 patients with HNC out of a total of 163 patients with a variety of cancers (over four different cancers were analyzed) compared to 13 healthy controls [62]. The results of this study indicated the difference between serum IGF-1/2 and IGFBP-2/3 levels in HNC patients before and after radiation therapy were insignificant, suggesting that they are ineffective prognostic markers for the efficacy of radiotherapy.

As discussed above, serum levels of the IGFs and IGFBPs are not a simple reflection of changes in secretion at the tumor level. IGFBP-3 is synthesized and secreted by many tissues and tumor types, as are IGF-1, IGF-2, and IGFBP-2. However, IGF-1 and IGFBP-2 are synthesized and secreted in large quantities by the liver, and this can mask the production by other sites. In a separate study, salivary levels of IGF-1, MMP-2 and MMP-9 were found to be higher in patients with oral cancer

compared to healthy age- and gender-matched controls [86]. While it is unclear what these elevated levels may be the result of pathologically, they suggest that one or more of these proteins may serve as an oral cancer biomarker. We have found that IGFBP-2 levels were reduced in human HNC tumor specimens compared to adjacent normal tissue [89]. This is similar to what was reported for IGFBP-5 in colonic myofibroblasts [38]. In the latter case, reduced IGFBP-5 levels resulted from enhanced MMP-mediated degradation. In our studies we have not validated whether reduced IGFBP-2 levels result from reduced expression or increased degradation.

6.5 IGF-1R Regulation by miR

There has been a significant upsurge in the study of microRNAs (miRs) and their roles in cellular dysregulation in cancer. miRs are 18–24 base pair RNAs that are not translated. Gene regulation via miRs is primarily through posttranscriptional binding to an mRNA target and prevention of translation. It has been suggested that as many as 800–1,000 miRs may exist [10], making this a fertile area for discovery in health and disease. miRs are key regulators of most cellular processes, ranging from cell motility, invasion, proliferation, differentiation, survival, and apoptosis. Of significance, miRs have been implicated as proto-oncogenes (oncomiRs) or as tumor suppressors (antagomiRs) and exhibit altered expression in different cancers, including HNC [54]. Making the landscape of oncogenesis even more complex, a single miR may bind to several targets both within a single pathway and across signaling events. Understanding which miRs can interact with the IGF pathway is paramount in understanding IGF dysregulation in cancer. Several miRs, including miR-7, miR-139, miR-145, and miR-675, have been identified as having a binding site in the IGF-1R 3’UTR and have subsequently been shown to affect IGF-1R levels within human tumor specimens, in vivo mouse models, and tumor cell lines (Fig. 6.1).

Jiang and coworkers recently analyzed miR-7 demonstrating its role in regulating IGF-1Rs in oral cancer cell lines [43]. Of note, a bioinformatics profiling of miR-7 targets revealed that in addition to the IGF-1R, additional signaling proteins targeted include IRS-1/2, the catalytic subunit of PI 3K, EGFR, raf-1, and PAK1 (p21 activated kinase 1). miR-7 was shown to suppress IGF-1R expression with a resultant decrease in proliferation and increase in apoptosis, suggesting a future role for miR-7 in HNC therapeutics. It will be important to establish miR-7 downregulation in HNC as reported in glioblastoma [45]. miR-7 is regulated via human antigen R (HuR), an RNA binding protein (RBP). This RBP also represses expression of miR-675, a miR associated with decreased cell proliferation during normal development. Keniry and colleagues recently showed that a decrease in miR-675 expression subsequently increases IGF-1R expression [46].

miR-145 also contributes to IGF-1R regulation in some cancers. A decrease in miR-145 expression has been observed in a variety of epithelial malignancies, and in hepatocellular carcinoma (HCC) in particular, it has been related to more aggressive cancer phenotypes and decreased life expectancy versus HCC patients without

miR-145 dysregulation [51]. Further, miR-145 binding regions in the 3'UTR of IRS-1 and -2 have been identified and verified using luciferase reporter assays. Inhibition of IGF-1R leading to MMP-2 downregulation via miR-139 upregulation has been studied. miR-139 downregulation leads to more invasive colorectal cancer phenotypes, although it does not seem to affect tumor proliferation [84]. Other miRs have been identified in HNC [103]. For example, miR-138 [44] reduces RhoC and ROCK levels, and miR-184 acts as an oncomiR. Therefore, it will be important to examine miRs that specifically target IGF-1 system components in HNC as well miRs that target effectors of IGF-1 and other growth factor signaling cascades. As one example, miR-222, which targets MMP-1 and manganese superoxide dismutase-2 to block cell invasion and metastasis, is lost in HNC [55]. In some malignancies, such as chronic lymphocytic leukemia, miR profiles have been established, leading to opportunities for targeted therapy [59]. Further understanding of miR circuitry and dysregulation in HNC is required in order to pursue a complete understanding of the malignant process and to identify potential therapeutic targets.

6.6 Therapeutic Targeting of IGF in Cancer

Given that reduced IGF-1R signaling has the potential of lowering cancer incidence in a variety of cancers, targeting this pathway may lead to decreased tumor growth and metastasis [40]. To this end, a number of molecular approaches have been developed for the targeting of the IGF system [76] (Table 6.1). Accordingly, the IGFBPs or IGFBP-mimetics may serve as therapeutics or lead compounds for developing small molecule IGF antagonists [75], [76]. Similarly, several experimental approaches for inhibiting primary tumor growth, including IGF-1R blocking antibodies, dominant negative mutants, antisense cDNA, and siRNA to downregulate IGF-1R expression have demonstrated the therapeutic potential of interfering with IGF-1R-mediated signaling in vivo, resulting in the introduction of NVP-AEW541 as the first small molecule IGF-1R TKI as a potential cancer therapeutic [30]. Of these potential therapies, antibodies to the IGF-1R, TKIs, and IGFBP-mimetics have been most intensely investigated.

The use of IGF-1R antibodies to specifically block the IGF-1R with minimal effect on the IR showed promise in early preclinical studies. Both cell culture and xenograft investigations illustrated a decrease in tumor growth [17], [40]. Recently, enthusiasm for these inhibitors has waned, or has been redirected, as clinical trials consistently showed very little to no response to single-drug treatment [23], [99]. The lack of efficacy is thought to be due to compensatory EGFR upregulation upon IGF-1R blockade [85]. New strategies aimed at blocking both IGF-1R and EGFR are underway [18] with coinhibition paired with radiation showing early promise [61].

Another class of therapeutics aimed at blocking IGF pathway signaling is the receptor tyrosine kinase inhibitors (RTKIs). Initial excitement over these compounds was reserved due to identical ATP binding sequences being present in the IGF-1R

Table 6.1 Drugs targeting the IGF system

(A) Anti-IGF-1R mAbs

Inhibitor	*Isotype*	*Company*	*Stage*
IMC-A12	fully human IgG1 (cixutumumab)	Imclone, Inc., NY, NY	Phase III Phase 1/II plus doxorubicin
AMG-479	fully human IgG1 (ganitumumab)	Amgen, Thousand Oaks, CA	Discontinued
AVE1642	humanized IgG1	Sanofi-Aventis, Paris, France	Discontinued
BIIB022	human non-glycosylated IgG4	Biogen Idec, Cambridge, MA	Phase I Phase I with sorafenib in HCC
CP-751,1871	fully human IgG2 (figitumumab)	Pfizer, NY, NY	Discontinued
MK-0646	humanized IgG1 (dalotuzumab)	Merck, Whitehouse Station, NJ	Phase III
R-1507	fully human IgG1 (robatumumab)	Roche, Basel, Switzerland	Discontinued
Sch717454	fully human IgG1, (19D12)	Schering-Plough, Kenilworth, NJ	Discontinued
h10H5	Mouse anti-human	Genentech, South San Francisco, CA	Preclinical
MM-141	Bispecific tetravalent antibody to IGF-1R & ErbB3	Merrimack Pharmaceuticals, Cambridge, MA	Phase I

(B) IGF-1R TKIs

Inhibitor	*Company*	*Stage*
AXL1717	Axelar, Stockholm, Sweden (picropodophyllin)	Phase I
BMS-754807	Bristol-Myers Squibb, Princeton, NJ	Phase I/II
INSM-18	Insmed, Richmond, VA; IGF-1R and HER2/Neu inhibitor) NDGA	Phase I/II
OSI-906	OSI Pharmaceuticals, Melville, NY, linsitnib	Phase I/II
NVP-AEW541	Novartis, Basel, Switzerland	Discontinued
XL-228	Exelixis, South San Francisco, CA, (IGF-1R, Src, FGFR, Bcr-Abl)	Preclinical
KW-2450	Kyowa Kirin	Phase I/II
ABDP	AstraZeneca	Preclinical
A-928605	Abbott	Preclinical
PL225B	Piramal Enterprises Limited	Phase I

(C) IGF-1R Anti-Sense Oligos

Inhibitor	*Company*	*Stage*
ATL1101	Antisense Therapeutics, LTD Afandin Pty, Ltd.	Preclinical

(D) IGF-1 and IGF-2 Ligand Sequestration Strategy

Inhibitor	*Company*	*Stage*
rhIGFBP3 (mecasermin rinfabate)	Insmed, Inc., Richmond, VA (formerly Celtrix) Iplex	Preclinical
MSDI-573	Medimmune	Phase II
BI836845	Boehringer Ingelheim	Phase I

and IR resulting in cotargeting of both receptors [40]. However, RTKIs were efficacious preclinically and cotargeting both the IGF1-R and IR may be advantageous in cancer, with the caveat that this inhibition must be tumor-cell specific [78]. Currently, at least two Phase II trials combining linsitinib with topotecan in patients with NSCLC are underway (NCT01533181 and NCT01387386).

The use of IGF-1/2 sequestering agents is another option for downregulation of IGF-1R signaling in cancer. IGFBPs are naturally occurring molecules that serve this function. Interestingly, the binding domain on different IGFBPs is highly variable, making drug design difficult [75]. Dysregulation of IGFBPs 2, 3, and 5 has been associated with cancer indicating that either introducing recombinant IGFBPs or rational drug design should focus on these species [76]. Our lab has explored IGFBP-2 as a lead molecule in inhibitory therapeutics [47], [73], [74]. Moreover, the application of IGFBP-2 as a cancer therapeutic is further strengthened by its impact on metabolic regulation [100]. The levels of IGFBP-2 and IGFBP-1 decrease in type 2 diabetes, as a result of hyperinsulinemia [12]. Compared to wild-type animals, IGFBP-2 transgenic mice exhibit a lean phenotype, are protected against developing age-related glucose intolerance, insulin resistance, and high blood pressure, and are resistant to developing obesity and insulin resistance when fed high-energy or high-fat diets [100]. In addition to their obesity resistance, these animals have reduced leptin levels, all of which suggest that IGFBP-2 may be a factor in obesity prevention [100]. A microarray analysis of leptin action revealed upregulation of IGFBP-2 in the livers of leptin versus vehicle-treated *ob/ob* mice [35]. This finding was further corroborated by acute IGFBP-2 overexpression in *ob/ob*, type 1 and type 2 diabetic mice, using adenoviral infection; in all cases plasma glucose and insulin levels were reduced. This improved glycemic control, which was independent of weight loss and food intake, was also observed in leptin-resistant animals, indicating that IGFBP-2 acts downstream of leptin action [35]. These findings provide further rationale for considering IGFBP-2 as a cancer therapeutic based on the reduced diabetic symptoms that IGFBP-2 therapy would likely provide. There is a growing literature showing that type 2 diabetes and cancer share many risk factors [32] and that drugs used to treat diabetes may reduce (e.g., metformin) or increase (e.g., glargine insulin) cancer risk. In addition to the increased risk posed by elevated IGFs, insulin, IGF-1R, and reduced IGF-2R levels in contributing to cancer incidence, reduced IGFBP-2 levels should also be considered [22]. While optimal therapeutic IGF targeting has not yet been attained, promising data is accumulating that with appropriate combination therapy and tumor biomarker targeting, IGF pathway disruption may be developed into a novel cancer therapeutic.

6.7 Summary and Perspective

With the introduction of molecularly targeted approaches to a variety of cancers including HNC, it is now possible to personally tailor each patient's therapy. The IGF-1R and its interactions with other growth factor systems lead to activation of

a multitude of downstream effectors, each serving as a potential target for HNC therapeutics. Optimal targeting of each activated system must be achieved in order to avoid tumor evasion of therapy. Much work remains to determine which therapies to use, and when, for the best patient outcomes. As basic scientists continue to map out the complex interactions of the tumor with its host, better therapeutic options will follow. With the identification of new regulators, such as miRs and access to next-generation sequencing platforms, it is likely that alternative approaches to personalized medicine will be designed. Targeting of the IGF system in HNC may prove to be an important benefactor of this approach.

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References

1. Ahmad T, Farnie G, Bundred NJ, Anderson NG. The mitogenic action of insulin-like growth factor I in normal human mammary epithelial cells requires the epidermal growth factor receptor tyrosine kinase. J Biol Chem. 2004;279:1713–9.
2. Alberobello AT, D'Esposito V, Marasco D, Doti N, Ruvo M, Bianco R, et al. Selective disruption of insulin-like growth factor-1 (IGF-1) signaling via phosphoinositide-dependent kinase-1 prevents the protective effect of IGF-1 on human cancer cell death. J Biol Chem. 2010;285:6563–72.
3. Bachelder RE, Lipscomb EA, Lin X, Wendt MA, Chadborn NH, Eickholt BJ, et al. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. Cancer Res. 2003;63:5230–3.
4. Barnes CJ, Ohshiro K, Rayala SK, El-Naggar AK, Kumar R. Insulin-like growth factor receptor as a therapeutic target in head and neck cancer. Clin Cancer Res. 2007;13:4291–9.
5. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res. 1995;55:249–52.
6. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B. The IGF-I receptor in cell growth, transformation and apoptosis. Biochimica et Biophysica Acta. 1997;1332:F105–26.
7. Baserga R. Customizing the targeting of IGF-1 receptor. Future Oncol. 2009a;5:43–50.
8. Baserga R. The insulin receptor substrate-1: a biomarker for cancer? Exp Cell Res. 2009b;315:727–32.
9. Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev. 2009;30:586–623.
10. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, et al. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet. 2005;37:766–70.
11. Burgaud JL, Baserga R. Intracellular transactivation of the insulin-like growth factor I receptor by an epidermal growth factor receptor. Exp Cell Res. 1996;223:412–19.
12. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer. 2004;4:579–91.
13. Canto M, Devesa SS. Oral cavity and pharynx cancer incidence rates in the United States. Oral Oncol. 2002;38:610–17.

14. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science. 1998;279:563–6.
15. Chang YS, Wang L, Suh YA, Mao L, Karpen SJ, Khuri FR, et al. Mechanisms underlying lack of insulin-like growth factor-binding protein-3 expression in non-small-cell lung cancer. Oncogene. 2004;23:6569–80.
16. Christofori G, Naik P, Hanahan D. Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during beta-cell tumorigenesis. Nat Genet. 1995;10: 196–201.
17. Clemmons DR. Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. Nat Rev Drug Discovery. 2007;6:821–33.
18. Croasdale R, Wartha K, Schanzer JM, Kuenkele KP, Ries C, Mayer K, et al. Development of tetravalent IgG1 dual targeting IGF-1R-EGFR antibodies with potent tumor inhibition. Arch Biochem Biophys. 2012;526:206–18.
19. Davies DE, Chamberlin SG. Targeting the epidermal growth factor receptor for therapy of carcinomas. Biochem Pharmacol. 1996;51:1101–10.
20. De Meyts P, Whittaker J. Structural biology of insulin and IGF1 receptors: implications for drug design. Nat Rev Drug Discov. 2002;1:769–83.
21. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell. 1991;64:849–59.
22. Djiogue S, Nwabo Kamdje AH, Vecchio L, Kipanyula MJ, Farahna M, Aldebasi YH, et al. Insulin resistance and cancer: the role of insulin and insulin-like growth factors. Endocr Relat Cancer. 2013;20:R1–R17.
23. Dolgin E. Drug companies look to biomarkers to salvage cancer target. Nat Med. 2012;18:1312–13.
24. El-Shewy HM, Johnson KR, Lee M-H, Jaffa AA, Obeid LM, Luttrell LM. Insulin-like growth factors mediate heterotrimeric G protein-dependent ERK1/2 activation by transactivating sphingosine 1-phosphate receptors. J Biol Chem. 2006;281:31399–407.
25. Endogenous H, Breast Cancer Collaborative Group. Key TJ, Appleby PN, Reeves GK, Roddam AW. Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: pooled individual data analysis of 17 prospective studies. Lancet Oncol. 2010;11:530–42.
26. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. 2002;23:824–54.
27. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, et al. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol. 1999;19:3278–88.
28. Friedrich RE, Hagel C, Bartel-Friedrich S. Insulin-like growth factor-1 receptor (IGF-1R) in primary and metastatic undifferentiated carcinoma of the head and neck: a possible target of immunotherapy. Anticancer Res. 2010;30:1641–43.
29. Fung C, Grandis JR. Emerging drugs to treat squamous cell carcinomas of the head and neck. Expert Opin Emerg Drugs. 2010;15:355–73.
30. Garcia-Echeverria C, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, et al. In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. Cancer Cell. 2004;5:231–9.
31. Gimona M, Buccione R, Courtneidge SA, Linder S. Assembly and biological role of podosomes and invadopodia. Curr Op Cell Biol. 2008;20:235–41.
32. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. CA Cancer J Clin. 2010;60:207–21.
33. Grandis JR, Chakraborty A, Zeng Q, Melhem MF, Tweardy DJ. Downmodulation of TGF-alpha protein expression with antisense oligonucleotides inhibits proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. J Cell Biochem. 1998;69:55–62.
34. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet. 1998;351:1393–6.

35. Hedbacker K, Birsoy K, Wysocki RW, Asilmaz E, Ahima RS, Farooqi IS, et al. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metab. 2010;11:11–22.
36. Hellstrom A, Perruzzi C, Ju M, Engstrom E, Hard AL, Liu JL, et al. Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. Proc Natl Acad Sci U S A. 2001;98:5804–8.
37. Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-Like Growth Factor Binding Protein-5 Is a Target of Matrix Metalloproteinase-7: Implications for Epithelial-Mesenchymal Signaling. Cancer Res. 2005a;65:7363–9.
38. Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res. 2005b;65:7363–9.
39. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol. 2005;23:1011–27.
40. Hofmann F, Garcia-Echeverria C. Blocking the insulin-like growth factor-I receptor as a strategy for targeting cancer. Drug Discov Today. 2005;10:1041–7.
41. Hung PS, Kao SY, Shih YH, Chiou SH, Liu CJ, Chang KW, et al. Insulin-like growth factor binding protein-5 (IGFBP-5) suppresses the tumourigenesis of head and neck squamous cell carcinoma. J Pathol. 2008;214:368–76.
42. Jameson MJ, Beckler AD, Taniguchi LE, Allak A, Vanwagner LB, Lee NG, et al. Activation of the insulin-like growth factor-1 receptor induces resistance to epidermal growth factor receptor antagonism in head and neck squamous carcinoma cells. Mol Cancer Ther. 2011;10:2124–34.
43. Jiang L, Liu X, Chen Z, Jin Y, Heidbreder CE, Kolokythas A, et al. MicroRNA-7 targets IGF1R (insulin-like growth factor 1 receptor) in tongue squamous cell carcinoma cells. Biochem J. 2010a;432:199–205.
44. Jiang L, Liu X, Kolokythas A, Yu J, Wang A, Heidbreder CE, et al. Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. Int J Cancer. 2010b;127:505–12.
45. Kefas B, Godlewski J, Comeau L, Li Y, Abounader R, Hawkinson M, et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res. 2008;68:3566–72.
46. Keniry A, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G, et al. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nat Cell Biol. 2012;14:659–65.
47. Kibbey MM, Jameson MJ, Eaton EM, Rosenzweig SA. Insulin-like growth factor binding protein-2: contributions of the C-terminal domain to insulin-like growth factor-1 binding. Mol Pharmacol. 2006;69:833–45.
48. Kim M, Gans JD, Nogueira C, Wang A, Paik JH, Feng B, et al. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. Cell. 2006;125:1269–81.
49. Knowlden JM, Hutcheson IR, Barrow D, Gee JMW, Nicholson RI. Insulin-like growth factor-I receptor signaling in tamoxifen-resistant breast cancer: a supporting role to the epidermal growth factor receptor. Endocrinology. 2005;146:4609–18.
50. Kuribayashi A, Kataoka K, Kurabayashi T, Miura M. Evidence that basal activity, but not transactivation, of the epidermal growth factor receptor tyrosine kinase is required for insulin-like growth factor I-induced activation of extracellular signal-regulated kinase in oral carcinoma cells. Endocrinology. 2004;145:4976–84.
51. Law PT, Ching AK, Chan AW, Wong QW, Wong CK, To KF, et al. MiR-145 modulates multiple components of the insulin-like growth factor pathway in hepatocellular carcinoma. Carcinogenesis. 2012;33:1134–41.
52. Levy-Toledano R, Blaettler DH, LaRochelle WJ, Taylor SI. Insulin-induced activation of phosphatidylinositol (PI) 3-kinase. Insulin-induced phosphorylation of insulin receptors and insulin receptor substrate-1 displaces phosphorylated platelet-derived growth factor receptors from binding sites on PI 3-kinase. J Biol Chem. 1995;270:30018–22.

53. Li S, Wang N, Brodt P. Metastatic cells can escape the proapoptotic effects of TNF-alpha through increased autocrine IL-6/STAT3 signaling. Cancer Res. 2012;72:865–75.
54. Liu X, Chen Z, Yu J, Xia J, Zhou X. MicroRNA profiling and head and neck cancer. Comp Funct Genomics. vol. 2009, Article ID 837514, 11 pages, 2009. doi:10.1155/2009/837514.
55. Liu X, Yu J, Jiang L, Wang A, Shi F, Ye H, et al. MicroRNA-222 regulates cell invasion by targeting matrix metalloproteinase 1 (MMP1) and manganese superoxide dismutase 2 (SOD2) in tongue squamous cell carcinoma cell lines. Cancer Genomics Proteomics. 2009b;6:131–9.
56. Lopez T, Hanahan D. Elevated levels of IGF-1 receptor convey invasive and metastatic capability in a mouse model of pancreatic islet tumorigenesis. Cancer Cell. 2002;1:339–53.
57. Loudig O, Brandwein-Gensler M, Kim RS, Lin J, Isayeva T, Liu C, et al. Illumina whole-genome complementary DNA-mediated annealing, selection, extension and ligation platform: assessing its performance in formalin-fixed, paraffin-embedded samples and identifying invasion pattern-related genes in oral squamous cell carcinoma. Hum Pathol. 2011;42:1911–22.
58. Lucas JT Jr, Salimath BP, Slomiany MG, Rosenzweig SA. Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent. Oncogene. 2010;29:4449–59.
59. Lujambio A, Lowe SW. The microcosmos of cancer. Nature. 2012;482:347–55.
60. Ma J, Pollak M, Giovannucc iE, Chan J, Tao Y, Hennekens C, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-1 and IGF-binding protein-3. J Natl Cancer Inst. 1999;91:620–5.
61. Matsumoto F, Valdecanas DN, Mason KA, Milas L, Ang KK, Raju U. The Impact of Timing of EGFR and IGF-1R Inhibition for Sensitizing Head and Neck Cancer to Radiation. Anticancer Res. 2012;32:3029–35.
62. Matuschek C, Rudoy M, Peiper M, Gerber PA, Hoff NP, Buhren BA, et al. Do insulin-like growth factor associated proteins qualify as a tumor marker? Results of a prospective study in 163 cancer patients. Eur J Med Res. 2011;16:451–6.
63. Mira E, Lacalle RA, Gonzalez MA, Gomez-Mouton C, Abad JL, Bernad A, et al. A role for chemokine receptor transactivation in growth factor signaling. EMBO Rep. 2001;2:151–6.
64. Natarajan M, Stewart JE, Golemis EA, Pugacheva EN, Alexandropoulos K, Cox BD, et al. HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. Oncogene. 2006;25:1721–32.
65. Novosyadlyy R, Vijayakumar A, Lann D, Fierz Y, Kurshan N, LeRoith D. Physical and functional interaction between polyoma virus middle T antigen and insulin and IGF-I receptors is required for oncogene activation and tumour initiation. Oncogene. 2009;28: 3477–86.
66. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem. 2002;277:39684–95.
67. Papadimitrakopoulou VA, Brown EN, Liu DD, El-Naggar AK, Jack Lee J, Hong WK, et al. The prognostic role of loss of insulin-like growth factor-binding protein-3 expression in head and neck carcinogenesis. Cancer Lett. 2006;239:136.
68. Pollak M. Targeting insulin and insulin-like growth factor signalling in oncology. Curr Opin Pharmacol. 2008a;8:384–92.
69. Pollak M. Targeting insulin and insulin-like growth factor signalling in oncology. Curr Opin Pharmacol. 2008b;8:384–92.
70. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nat Rev Cancer. 2012;12:159–69.
71. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer. 2004;4:505–18.
72. Punglia R, Lu M, Hsu J, Kuroki M, Tolentino J, Keough K, et al. Regulation of VEGF expression by IGF-1. Diabetes. 1997;46:1619–26.
73. Robinson SA, Rosenzweig SA. Synthesis and characterization of biotinylated forms of insulin-like growth factor-1: topographical evaluation of the IGF-1/IGFBP-2 AND IGFBP-3 interface. Biochemistry. 2004;43:11533–45.

74. Robinson SA, Rosenzweig SA. Paradoxical effects of the phage display-derived peptide antagonist IGF-F1-1 on insulin-like growth factor-1 receptor signaling. Biochem Pharmacol. 2006;72:53–61.
75. Rosenzweig SA. What's new in the IGF-binding proteins? Growth Horm IGF Res. 2004;14:329–36.
76. Rosenzweig SA, Atreya HS. Defining the pathway to insulin-like growth factor system targeting in cancer. Biochem Pharmacol. 2010;80:1115–24.
77. Roudabush FL, Pierce KL, Maudsley S, Khan KD, Luttrell LM. Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. J Biol Chem. 2000;275:22583–9.
78. Sachdev D, Yee D. Disrupting insulin-like growth factor signaling as a potential cancer therapy. Mol Cancer Ther. 2007;6:1–12.
79. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 1995;19:183–232.
80. Schmitz S, Kaminsky-Forrett MC, Henry S, Zanetta S, Geoffrois L, Bompas E, et al. Phase II study of figitumumab in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck: clinical activity and molecular response (GORTEC 2008-02). Ann Oncol. 2012;23:2153–61.
81. Sciacca L, Prisco M, Wu A, Belfiore A, Vigneri R, Baserga R. Signaling differences from the A and B isoforms of the insulin receptor (IR) in 32D cells in the presence or absence of IR substrate-1. Endocrinology. 2003;144:2650–8.
82. Sell C, Rubini M, Rubin R, Liu J, Efstratiadis A, Baserga R. Simian Virus 40 Large Tumor Antigen is Unable to Transform Mouse Embryonic Fibroblasts Lacking Type 1 Insulin-Like Growth Factor Receptor. Proc Natl Acad Sci USA. 1993;90:11217–21.
83. Shaw LM. Identification of insulin receptor substrate 1 (IRS-1) and IRS-2 as signaling intermediates in the {alpha}6{beta}4 integrin-dependent activation of phosphoinositide 3-OH kinase and promotion of invasion. Mol Cell Biol. 2001;21:5082–93.
84. Shen K, Liang Q, Xu K, Cui D, Jiang L, Yin P, et al. MiR-139 inhibits invasion and metastasis of colorectal cancer by targeting the type I insulin-like growth factor receptor. Biochem Pharmacol. 2012;84:320–30.
85. Shin DH, Min HY, El-Naggar AK, Lippman SM, Glisson B, Lee HY. Akt/mTOR counteract the antitumor activities of cixutumumab, an anti-insulin-like growth factor I receptor monoclonal antibody. Mol Cancer Ther. 2011;10:2437–48.
86. Shpitzer T, Bahar G, Feinmesser R, Nagler RM. A comprehensive salivary analysis for oral cancer diagnosis. J Cancer Res Clin Oncol. 2007;133:613–7.
87. Slomiany MG, Rosenzweig SA. Hypoxia-inducible factor-1-dependent and -independent regulation of insulin-like growth factor-1-stimulated vascular endothelial growth factor secretion. J Pharmacol Exp Ther. 2006;318:666–75.
88. Slomiany MG, Black LA, Kibbey MM, Day TA, Rosenzweig SA. IGF-1 induced vascular endothelial growth factor secretion in head and neck squamous cell carcinoma. Biochem Biophys Res Commun. 2006;342:851–8.
89. Slomiany MG, Black LA, Kibbey MM, Tingler MA, Day TA, Rosenzweig SA. Insulin-like growth factor-1 receptor and ligand targeting in head and neck squamous cell carcinoma. Cancer Lett. 2007;248:269–79.
90. Smith LE, Shen W, Perruzzi C, Soker S, Kinose F, Xu X, et al. Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-1 receptor. Nat Med. 1999;5:1390–5.
91. Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc Natl Acad Sci U S A. 2004;101:2076–81.
92. Stoica A, Saceda M, Fakhro A, Joyner M, Martin MB. Role of insulin-like growth factor-I in regulating estrogen receptor-alpha gene expression. J Cell Biochem. 2000;76:605–14.

93. Strnad H, Lacina L, Kolar M, Cada Z, Vlcek C, Dvorankova B, et al. Head and neck squamous cancer stromal fibroblasts produce growth factors influencing phenotype of normal human keratinocytes. Histochem Cell Biol. 2010;133:201–11.
94. Tamburini J, Chapuis N, Bardet V, Park S, Sujobert P, Willems L, et al. mTORC1 inhibition activates PI3K/Akt by up-regulating IGF-1R signaling in acute myeloid leukemia: rational for therapeutic inhibition of both pathways. Blood. 2008;111:379–82.
95. Tao Y, Pinzi V, Bourhis J, Deutsch E. Mechanisms of disease: signaling of the insulin-like growth factor 1 receptor pathway—therapeutic perspectives in cancer. Nat Clin Pract Oncol. 2007;4:591–602.
96. Tikhmyanova N, Little JL, Golemis EA. CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci. 2010;67:1025–48.
97. Ueda S, Tsuda H, Sato K, Takeuchi H, Shigekawa T, Matsubara O, et al. Alternative tyrosine phosphorylation of signaling kinases according to hormone receptor status in breast cancer overexpressing the insulin-like growth factor receptor type 1. Cancer Sci. 2006;97:597–604.
98. Weber A, Hengge UR, Stricker I, Tischoff I, Markwart A, Anhalt K, et al. Protein microarrays for the detection of biomarkers in head and neck squamous cell carcinomas. Hum Pathol. 2007;38:228–38.
99. Weroha SJ, Haluska P. IGF-1 receptor inhibitors in clinical trials—early lessons. J Mammary Gland Biol Neoplasia. 2008;13:471–83.
100. Wheatcroft SB, Kearney MT, Shah AM, Ezzat VA, Miell JR, Modo M, et al. IGF-binding protein-2 protects against the development of obesity and insulin resistance. Diabetes. 2007;56:285–94.
101. Whittaker J, Whittaker LJ, Roberts CT, Jr., Phillips NB, Ismail-Beigi F, Lawrence MC, et al. alpha-Helical element at the hormone-binding surface of the insulin receptor functions as a signaling element to activate its tyrosine kinase. Proc Natl Acad Sci U S A. 2012;109:11166–71.
102. Wirth LJ, Haddad RI, Lindeman NI, Zhao X, Lee JC, Joshi VA, et al. Phase I study of gefitinib plus celecoxib in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol. 2005;23:6976–81.
103. Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue. Clin Cancer Res. 2008;14:2588–92.
104. Wu X, Zhao H, Do K-A, Johnson MM, Dong Q, Hong WK, et al. Serum levels of insulin growth factor (IGF-I) and IGF-binding protein predict risk of second primary tumors in patients with head and neck cancer. Clin Cancer Res. 2004a;10:3988–95.
105. Wu X, Zhao H, Do KA, Johnson MM, Dong Q, Hong WK, et al. Serum levels of insulin growth factor (IGF-I) and IGF-binding protein predict risk of second primary tumors in patients with head and neck cancer. Clin Cancer Res. 2004b;10:3988–95. (an official journal of the American Association for Cancer Research).
106. Yang Y, Yee D. Targeting insulin and insulin-like growth factor signaling in breast cancer. J Mammary Gland Biol Neoplasia. 2012;17:251–61.
107. Yu H, Spitz MR, Mistry J, Gu J, Hong WK, Wu X. Plasma levels of insulin-like growth factor-I and lung cancer risk: a case-control analysis. J Natl Cancer Inst. 1999;91:151–6.
108. Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, et al. Gene expression profiles in normal and cancer cells. Science. 1997;276:1268–72.
109. Zhang Q, Thomas SM, Lui VWY, Xi S, Siegfried JM, Fan H, et al. Phosphorylation of TNF-{alpha} converting enzyme by gastrin-releasing peptide induces amphiregulin release and EGF receptor activation. Proc Natl Acad Sci. 2006;103:6901–06.

Chapter 7
The PI3K Signaling Pathway in Head and Neck Squamous Cell Carcinoma

Jason D. Howard and Christine H. Chung

Abstract The PI3K/PTEN/AKT/mTOR signaling axis has been intensively studied in many cancer systems. Current evidence suggests deregulation of this pathway plays a unique role in the initiation, development, and recurrence of head and neck squamous cell carcinoma (HNSCC). A heterogeneous disease by nature, HNSCC encompasses a disparate collection of anatomical sites with complex tumor biology. Yet, PI3K/PTEN/AKT/mTOR signaling has an intimate role in nearly every facet of this disease. In this chapter, we will provide a brief introduction to the mechanisms involved in PI3K/PTEN/AKT/mTOR signaling and how specific alterations in these signaling nodes enable HNSCC development. We will also discuss differences in PI3K/PTEN/AKT/mTOR signaling with respect to HPV status. A number of inhibitors targeting multiple nodes in this pathway have been developed as agents have broad application across many cancer types. We will briefly review how these therapeutic agents are being evaluated and what predictive biomarkers have been established in HNSCC for these drugs. Finally, PI3K/PTEN/AKT/mTOR signaling represents an important source of resistance to radiation, chemotherapy, and other targeted agents. We will also speculate on how PI3K/PTEN/AKT/mTOR inhibitors may increase the efficacy of these established therapies. Although PI3K/PTEN/AKT/mTOR investigations are relatively new to HNSCC research, early evidence suggests further evaluation of this essential signal transduction pathway is warranted.

Keywords PI3K · PTEN · AKT · mTOR · HPV · HNSCC · Biomarkers

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7.1 Introduction

The PI3K/PTEN/AKT/mTOR pathway is a critical signaling axis which consolidates and regulates the myriad extracellular signals required for complex, multicellular organisms. The end result of appropriate PI3K/PTEN/AKT/mTOR signaling is homeostasis: the careful balance of proliferation, metabolism, autophagy, cap-dependent translation, migration, apoptosis, and many other cellular requirements. Given the magnitude of functionalities associated with this pathway, deregulation at any of its signaling nodes can have dire biological consequences. Thus, many of the proteins in this pathway have been established as bona fide oncogenes or tumor suppressors. Recent evidence suggests that at least 47 % of head and neck squamous cell carcinomas (HNSCCs) have at least one molecular alteration in this pathway [1]. In this chapter, we will summarize key features of this pathway, and how these molecular alterations are associated with HNSCC development and progression. In addition, we will provide some perspective regarding the translational potential of known therapeutic targets involved in this signaling network and development of biomarkers for assessing clinical outcomes. Ideally, these targeted agents would ultimately exploit an oncogenic dependence unique to HNSCC, or subvert acquired resistance mechanisms mediated by PI3K/AKT/mTOR signaling to enhance the efficacy of previously established therapies.

7.2 PI3K/PTEN/AKT/mTOR Signal Transduction

7.2.1 Phosphoinositide 3-kinase (PI3K)

The intracellular transduction of extracellular stimuli often requires receptor-mediated signaling. Thus, membrane-bound receptors translate extracellular ligand binding into intracellular signaling cascades to various downstream cellular compartments (Fig. 7.1). Adaptor proteins and second messengers play an important role in correctly mediating and regulating these signals. One group of second messengers is the class I phosphoinositide 3-kinase (PI3K) family (p110α, p110β, p110γ, and p110δ), a common signaling mechanism utilized by a wide array of receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). A functional PI3K-signaling unit contains one regulatory (typically p85) and one catalytic (p110) protein, creating a heterodimeric kinase with enzymatic activity for lipid and protein substrates [2], [3]. However, only the lipid kinase activity is required for oncogenic signaling [4]. When a receptor is activated, PI3K translocates to the cell membrane where it associates with the receptor through p85 and various adaptor proteins (i.e., IRS1) [5], [6]. This binding relinquishes p85-negative regulation of p110, initiating catalytic activity. PI3K can also be positively affected by Ras, a critical GTPase which may facilitate PI3K membrane localization [7]–[9].

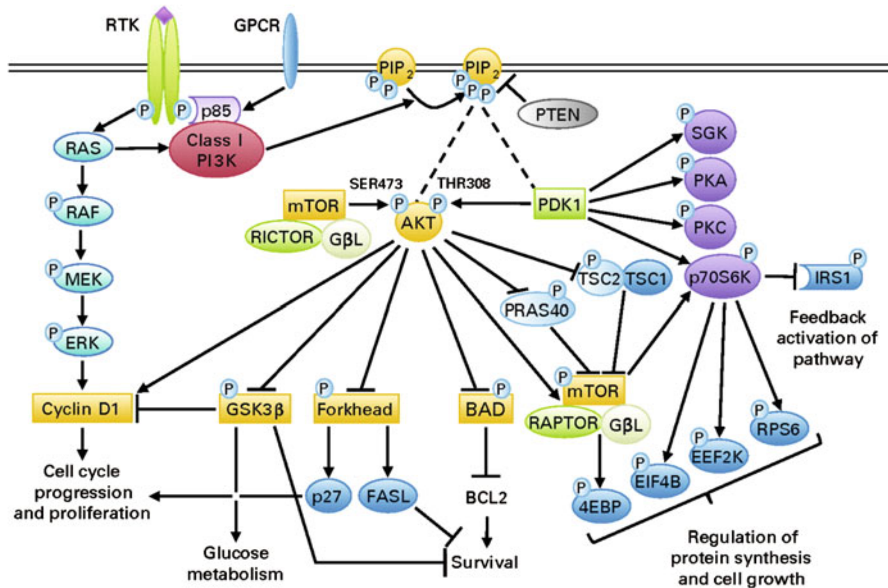

Fig. 7.1 Schematic diagram of the PI3K/PTEN/AKT/mTOR pathway. (Reprinted with permission, ©2012 American Society of Clinical Oncology. All rights reserved [181])

Once active, PI3K catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 then serves to localize AKT and its activating kinase, 3-phosphoinositide-dependent kinase 1 (PDK1), to the cell membrane (Fig. 7.1). Following PDK1-mediated AKT activation at threonine 308 (T308) [10], AKT exerts considerable downstream effects on transcription, protein synthesis, metabolism, proliferation, and apoptosis. Aside from the protein phosphatases which carefully balance the activity of these kinases, the pathway is also negatively regulated by phosphatase and tensin homologue (PTEN), which catalyzes the dephosphorylation of PIP_3 to PIP_2 [11], [12].

Investigations of oncogenic PI3K have been focused largely on p110α (*PIK3CA*). Exome sequencing projects have determined this isoform represents the vast majority of cancer-related PI3K mutations [13]. It is currently estimated that p110α is mutated in 6–20 % of HNSCCs [14]–[17]. Unlike tumor suppressors, these mutations are not spread throughout the gene. Accordingly, 80 % of these cancer-associated modifications occur within three “hot spot” locations: E542, E545, and H1047. The first two mutations decouple p110 from p85, releasing the inhibitory effect of the regulatory subunit [18], [19]. The third mutation introduces a conformational change in the activation loop [18], possibly mimicking Ras-mediated activation [20]. Other cancer-specific mutations do exist within the gene; however, they have lower oncogenic activity and provide less of a selective advantage for tumorigenesis [21], [22].

7.2.2 PTEN

PTEN is a critical tumor suppressor, originally discovered because complete or partial deletion of chromosome 10 is a common event in a number of cancers, including brain, bladder, and prostate [23]. At least 80 % of Cowden's disease patients harbor heritable, germline mutations in PTEN which confer a rare familial cancer syndrome [24]–[26]. Although mostly known for catalyzing the reaction of PIP_3 back to PIP_2, this gene encodes a protein which possesses both peptide and phospholipid phosphatase activity (Fig. 7.1; [11], [12]). Loss of PTEN function causes an accumulation of PIP_3 at the cell membrane. This enriched pool of PIP_3 recruits AKT/mTOR pathway members (AKT isoforms, PDK1, etc.) to the cell membrane and inappropriately initiates the activation of this central signaling axis.

Knockout experiments have determined that PTEN, while essential for viable development, also has tumor suppressive functions in endometrial, liver, prostate, gastrointestinal, thyroid, and thymus tissues [27]. Haploinsufficiency is often sufficient to mediate a loss of PTEN function [28], [29]. Due to its singular importance, PTEN function is regulated, and consequently deregulated, by a myriad of mechanisms: mutation, deletion, epigenetic silencing, transcriptional, post-transcriptional, and microRNA (miRNA) regulation, post-translational modification, and various protein–protein interactions. The effect of PTEN on PI3K/AKT/mTOR pathway activity is well established; however, multiple tumor suppressors exist within this pathway. As PTEN is the most frequently deregulated tumor suppressor associated with this pathway, additional functions independent of PI3K/AKT/mTOR likely imbue PTEN with added functional importance. For example, a loss of PTEN causes PIP_2 depletion, an important membrane-associated regulator of cell polarity. This morphological modulation initiates a loss of epithelial characteristics, similar to epithelial-to-mesenchymal transition (EMT) [30], a hallmark of particularly aggressive cancers. Following EMT, neoplastic cells have increased cell motility and are often more resistant to standard therapy (reviewed in [31]). PTEN also localizes to the nucleus and is involved in maintaining chromosomal stability. Consequently, a loss of nuclear PTEN enhances chromosomal instability and leads to spontaneous DNA double-strand breaks [32]. Furthermore, PTEN only exhibits PIP_3 phosphatase activity in the cytoplasm, thus PTEN may affect genomic stability and cell cycle progression in the nucleus by lipid phosphatase-independent mechanisms [33]. Due to its numerous PI3K-dependent and -independent functions, PTEN is widely considered a critical tumor suppressor with loss of function often resulting in cancer.

7.2.3 AKT

AKT, also known as protein kinase B (PKB), is a critical node for mammalian signal transduction and the major effector of PI3K signaling. This vital serine/threonine protein kinase was originally discovered as the human homolog of *v-akt*, an oncogene transduced by the murine retrovirus AKT8 [34]–[37]. The AKT family is represented

by three isoforms: AKT1, AKT2, and AKT3. AKT1 is ubiquitously expressed at high levels [36]–[38], while the remaining isoforms are expressed in a more tissue-specific manner. Insulin-sensitive cells, such as liver, skeletal muscle, and adipose tissue demonstrate high levels of AKT2 expression [39], [40]. Meanwhile, AKT3 is highly expressed in the brain and testes, with lower levels of expression observed in muscle and intestinal organs [41]. While cancer-related AKT research largely focuses on AKT1, large-scale cancer sequencing projects have uncovered single nucleotide polymorphisms (SNPs) and somatic mutations associated with AKT2 and AKT3 [42], [43]. Mouse knockout models of the various AKT isoforms demonstrate specific mutant phenotypes, but are all viable [44]–[48]. Thus, the lack of embryonic lethality suggests that while each AKT isoform has characteristic signaling functions, they share a degree of functional compensation.

AKT kinases are comprised of an N-terminal pleckstrin homology (PH) domain, a flexible linker, and a C-terminal catalytic domain. While PIP_3 interacts with AKT via the PH domain [49], AKT is phosphorylated by PDK1 on the C-terminal activation loop (T308) and at serine 473 (S473) by mTORC2 (Fig. 7.1; [50]) to achieve full kinase activity [51]. While these mechanisms represent canonical AKT activation, a number of PIP_3-independent mechanisms also initiate AKT signaling. Activated CDC42 kinase 1 (Ack1 or TNK2) [52], [53], Src [54], protein-tyrosine kinase 6 (PTK6) [55], and serine/threonine-protein kinase 1 (TBK1) [56]–[58] all possess the ability to modulate AKT activity by noncanonical means. Once activated, AKT phosphorylates downstream targets altering cell survival, growth, proliferation, metabolism, and crosstalk with other signaling pathways. The most important downstream target of AKT is mammalian target of rapamycin (mTOR), a master regulator of cell growth, metabolism, translation initiation, and ribosome biogenesis. AKT also affects cell survival by negatively regulating proapoptotic proteins such as FOXO and MDM2, a negative regulator of p53 [59], [60]. AKT can also enhance cell-cycle turnover by phosphorylating glycogen synthase kinase 3 (GSK-3), which stabilizes cyclin D/E, c-jun, and c-myc proteins [61]–[64].

Recent evidence suggests that subcellular localization is an important determinant of AKT activity and downstream signaling. In fact, two important AKT substrates (FOXO proteins and p300) are sequestered solely in the nucleus [65], [66]. Despite lacking a nuclear localization signal, AKT likely translocates to the nucleus by interacting with members of the T-cell leukemia-1 (TCL1) family of oncoproteins. These proteins are capable of complexing with AKT to serve as coactivators, shuttling AKT to the nucleus [67], [68]. Increased nuclear phospho-AKT has been observed in acute myeloid leukemia [69], [70], lung [71], breast [72], thyroid [73], and prostate cancers [74]. Nuclear phospho-AKT detection has also been positively correlated with prostate cancer progression [74] and Gleason score [75]. Nuclear AKT activity may have specific oncogenic effects as promyelocytic leukemia protein (PML), which functions to dephosphorylate AKT within the nucleus and is a known tumor suppressor [76].

Due to the staggering number of pathways dependent on AKT signaling, deregulation of this enzyme by alterations in associative proteins or changes in subcellular

localization can have disastrous biological consequences. For example, mosaic expression of an AKT point mutant (AKT E17K) is responsible for almost 90 % of Proteus syndrome cases, the debilitating growth disorder suffered by Joseph Merrick, popularly known as "The Elephant Man" [77]. Proteus syndrome is characterized by segmental overgrowth and hyperplasia of a variety of tissues and organs, which also includes an increased risk of tumorigenesis [78], [79]. The rare nature of this crippling disease (< 1 case/1 million) lies in its dependence on mosaic expression, as constitutive somatic or germline expression of this mutant is lethal. Not surprisingly, this mutation has been detected in a variety of cancers including breast [80], urinary tract [81], and endometrial cancers [82]. Although the incidence of AKT E17K in patient tumor samples is low (1–4 %), it nonetheless represents an important component of the total PI3K/PTEN/AKT/mTOR deregulation that occurs during tumorigenesis.

7.2.4 mTOR

As mentioned above, mTOR is the single most important effector of AKT signaling. Serving as the catalytic subunit of two macromolecular complexes (mTORC1 and mTORC2), mTOR is a master regulator of cell growth. Although mTOR is shared between these two complexes, the associative proteins unique to each tune the activity of this enzyme for distinct substrates and sources of regulation [83]–[89]. mTORC1 consists of mTOR, Deptor, Raptor, mLST8, and PRAS40 [90], [91]. This complex is rapamycin sensitive [92]–[94], and S6K1 and 4E-BP1 are its most important downstream targets (Fig. 7.1; [95]–[97]). Phosphorylation of S6K1 promotes mRNA translation by facilitating initiation and elongation complex formation at the mRNA transcript. Activation of 4E-BP1 allows eIF4E to recruit eIF4G and initiate 5′ mRNA translation. Aside from protein synthesis, mTORC1 also regulates ribosome biogenesis and autophagy [98]–[100]. Recent studies have shown that mTORC1 activation is sufficient to inhibit autophagy, which is reversible following mTORC1 inhibition [101].

mTORC2 also contains Deptor and mLST8; however, additional associative proteins include Rictor, mSIN1, and Protor [89], [102], [103]. Differential phosphorylation of AKT (T308 vs. S473) had long been understood, with PDK1 mediating T308 activation. However, it was recently discovered that mTORC2 is the complex responsible for "PDK2" activity, phosphorylating AKT at S473 [50]. Consequently, this functionality places mTORC2 in a positive feedback loop within the pathway, allowing AKT to achieve full activation. This function was initially difficult to elucidate as mTORC2 is rapamycin insensitive during acute treatment [92]–[94]. Along with AKT, mTORC2 can also activate serum- and glucocorticoid-regulated kinase (SGK) and protein kinase C (PKC) [50], [104]–[106].

Because mTOR has a central role in controlling cell growth, appropriate regulation of mTOR itself is paramount to maintaining homeostasis. Thus, it is not surprising

that a number of familial cancer syndromes involve germline mutations of mTOR-negative regulators (Cowden disease, tuberous sclerosis) [26], [107]. Transgenic mice have also provided experimental evidence for the importance of appropriate mTOR regulation. Mice heterozygous for beclin or autophagy-related 4C (ATG4C), both critical regulators of autophagy, are prone to tumor formation due to defects in autophagosome formation [108]–[110]. As a negative regulator of autophagy, sustained mTORC1 activation has the ability to mimic these genetic modifications and enhance tumor development. Sustained mTORC2 activity is also capable of driving tumorigenesis through constitutive activation of AKT and SGK. Furthermore, expression of Rictor is required for tumor cell line and prostate tumor growth in PTEN-deficient mice [111], [112]. Consequently, tumor-associated defects in PI3K, PTEN, or AKT all have the potential to initiate pathological mTOR signaling. However, multiple routes of deregulation may provide important biomarkers and potential targets of therapeutic intervention to alleviate the oncogenic effects of mTOR signaling in HNSCC.

7.3 PI3K/PTEN/AKT/mTOR Deregulation in HNSCC

7.3.1 Genetic Alterations of PIK3CA in HNSCC

PI3K functions with critical importance to potentiate and regulate receptor-mediated extracellular stimuli. This vital second messenger has been intensively studied in cancer progression, including in HNSCC. PI3K, and more specifically *PIK3CA*, is a bona fide oncogene in HNSCC. As mentioned above, *PIK3CA* contains activating point mutations (commonly E542, E545, or H1047) in 6–20 % of HNSCC tumor samples (Table 7.1; [14], [15]). In fact, two sequencing projects independently identified *PIK3CA* as a significantly mutated oncogene in HNSCC tumor samples [16], [17]. Stransky et al. determined 8 % (6/74) of their tumor samples had *PIK3CA*-activating mutations: 1-R115 L (rare), 3-E542–545, 2-H1047 [16]. Agrawal et al. reported 6 % of their tumors harbored *PIK3CA* mutations: all three H1047 mutants [17]. In addition to these activating point mutations, copy number gains within the *PIK3CA* locus (3q26) are extremely common [113].

Current evidence suggests *PIK3CA* copy number gain is an early event in HNSCC development. Oral premalignant lesions commonly demonstrate an increase in *PIK3CA* copy number (39 %) [1]. An equivalent incidence of *PIK3CA* copy number gain is also noted in HNSCC tumors (32–37 %) [1], [114]. Along with alterations in ERK/MAPK, fibroblast growth factor (FGF), and p53, deregulated PTEN and PI3K/AKT pathway members delineate high-grade premalignant lesions from low-grade dysplasias [115]. Increased *PIK3CA* copy number is also associated with early HNSCC recurrence, but this difference is only statistically significant in patients without lymph node metastases ($p = 0.026$) [114].

Table 7.1 Investigations of PI3K/PTEN/AKT/mTOR genetic alterations in premalignant lesions and squamous cell carcinoma of head and neck

Pathway member	Investigator	Samples	Methods	Result	Reference
PIK3CA	Redon et al.	45 HNSCC tumors (no nodal involvement)	CGH, FISH	60 % 3q26 gain (PIK3CA locus)	182
	Woenckhaus et al.	15 PMLs 15 HNSCC tumors	FISH	3q26 CN gain low to mod. PML: 1/6 high-grade PML: 7/9 HNSCC:11/11 3q26 amp low to mod. PML:0/6 High-grade PML: 1/7 HNSCC: 6/11	113
	Pedrero et al.	38 PMLs 117 HNSCC tumors	qRT-PCR	Amplified 39 % PMLs Amplified 37% HNSCC tumors	1
	Sticht et al.	280 HNSCC TMA	FISH	39 % CN gain	183
	Qiu et al.	30 HNSCC tumors 8 HNSCC cell lines	gPCR/sequencing	Total mutated samples: 11 %	14
	Fenic et al.	33 HNSCC tumors	qRT-PCR gPCR/sequencing	36.4 % CN gain 9 % amplified 48.5 % mRNA overexpression No mutations detected	184
	Murugan et al.	37 HNSCC tumors 17 HNSCC cell lines	gPCR/sequencing	Mutant cell lines: 29.4 % Mutant tumors: 10.5 %	15
	Pattje et al.	140 HNSCC tumors	IHC	High p110α: 41 %	163
	Stransky et al.	74 HNSCC tumors	WES	Mutant tumors: 8 %	16
	Agrawal et al.	120 HNSCC tumors	WES, gPCR/sequencing	Mutant tumors: 6 %	17
	Kondo et al.	16 HNSCC cell lines	gPCR/sequencing	Mutant cell lines: 12.5 %	185
	Morris et al.	31 HNSCC tumors	CGH gPCR/sequencing	45.2 % CN gain Mutant tumors: 6 %	186
	Suda et al.	115 HNSCC tumors	qRT-PCR, CGH gPCR/sequencing	32.2 % CN gain Mutant tumors: 2.6 %	114
PTEN	Shao et al.	19 HNSCC tumors	PCR, gPCR/sequencing	LOH: 40 %, mutant tumors 16 %	116
	Lee et al.	41 HNSCC tumors	IHC	PTEN negative: 29 %	117
	Pedrero et al.	117 HNSCC tumors	MPA, PCR	LOH: 14 %, no detectable homozygous deletions	1

Table 7.1 (continued)

Pathway member	Investigator	Samples	Methods	Result	Reference
	Nathan et al.	22 HNSCC tumors	FISH	Tumor PTEN abnormalities: 68 %	187
		23 tumor-free margins		Margin PTEN abnormalities: 35 %	
	Murugan et al.	37 HNSCC tumors	gPCR/sequencing	No mutations detected	15
		17 HNSCC cell lines			
	Pattje et al.	140 HNSCC tumors	IHC	PTEN low expression: 69 %	163
	Stransky et al.	74 HNSCC tumors	WES	Mutant tumors: 7 %	16
	Kondo et al.	16 HNSCC cell lines	gPCR/sequencing	Mutant cell lines: 12.5 %	185
	Snietura et al.	147 HNSCC tumors	IHC	PTEN low expression: 59.9 %	164
AKT	Gupta et al.	38 HNSCC tumors	IHC	2–3+ pAKT staining: 65.8 %	160
	Pedrero et al.	117 HNSCC tumors	qRT-PCR, WB	pAKT detected: 17 %	1
				AKT2 amplification: 30 %	
	Molinolo et al.	305 HNSCC TMA	IHC	pAKT473 0: 1 %, 1–2: 31 %, 3–4: 66 %	188
				pAKT308 0: 4 %, 1–2: 57 %, 3–4: 42 %	
mTOR	Clark et al.	72 HNSCC tumors	IHC	p-mTOR detected: 59 %	189
	Morris et al.	31 HNSCC tumors	CGH	Rictor CN gain: 38.7 %	186
Downstream Targets	Nathan et al.	27 Tumor-free margins	IHC	eIF4E overexpressed: 70 %	162
				All eIF4E overexpressing tumors	
				Exhibited high p-p70S6K	
	Molinolo et al.	305 HNSCC TMA	IHC	pS6 0: 2 %, 1–2: 13 %, 3–4: 79 %	188
	Clark et al.	72 HNSCC tumors	IHC	p-4EBP1: 36 %	189

CGH comparitive genomic hybridization, *FISH* fluorescent in situ hybridization, *PML* premalignant lesion, *CN* copy number, *qRT-PCR* quantitative real-time polymerase chain reaction, *TMA* tissue microarray, *gPCR* genomic PCR, *IHC* immunohistochemistry, *WES* whole-exome sequencing, *LOH* loss of heterozygosity, *MPA* microsatellite pattern analysis, *pAKT* phospho-AKT, *WB* Western blot, *p-mTOR* phospho-mTOR, *p-p70S6K* phospho-p70S6K, *p-S6* phospho-S6, *p-4EBP1* phospho-4EBP1

7.3.2 PTEN Loss

A loss of PI3K-negative regulation has been observed in a number of independent HNSCC studies, as alterations in PTEN status are common (Table 7.1). Early efforts to catalog PTEN deregulation in HNSCC began with a screen of 19 tumors, which determined PTEN was mutated in three samples [116]. A loss of heterozygosity (LOH) also occurred within the PTEN locus (10q23) in 6 of the 15 evaluable samples. Within the mutant PTEN patients, two had stage IV disease while the third had recurrent, metastatic and stage III disease. In a larger study, targeted analysis of PI3K/AKT/mTOR HNSCC genetic alterations detected PTEN LOH in 14 % of the samples [1]. Three of the eight patients with PTEN LOH also demonstrated abnormal PTEN levels in the adjacent mucosa, suggesting both *PIK3CA* and PTEN deregulation are early events in HNSCC development. An additional investigation in squamous cell carcinoma of the tongue determined PTEN loss was evident in 29 % of the tumor samples [117]. Deregulated PTEN also correlated with decreased overall survival ($p = 0.03$) and event-free survival ($p = 0.01$). While these studies were targeted in nature, PTEN loss was also evident in one of the two HNSCC genome sequencing projects referenced above. Stransky et al. detected PTEN mutations in 7 % of their tumor samples [16], while PTEN abnormalities were not detected by Agrawal et al. [17].

Although LOH and PTEN mutation have been described in many cancer systems, protein loss by miRNA deregulation is a relatively new field of study. These short, noncoding RNAs are capable of regulating a wide variety of proteins, and thus represent oncogenes and tumor suppressors in their own right. A recent study of HNSCC tumor samples and cell lines determined miR-21 is overexpressed with respect to normal tissue [118]. miR-21 overexpression downregulated HNSCC PTEN protein levels *in vitro*, activated phospho-AKT, and increased the proliferation of immortalized keratinocytes (HaCaT) [118]. Consequently, miR-21 has been described as a proto-oncogene in HNSCC. However, miR-21 is not the only miRNA capable of targeting PTEN protein expression. miR-9 is a frequently methylated gene in HNSCC tumor samples, with miR-9 expression levels closely correlating with methylation status [119]. When miR-9 is reexpressed with the use of a demethylating agent, a significant increase in PTEN and a concomitant decrease in cell growth is observed [119]. While the connection between miR-9 and PTEN is indirect, this study does provide additional evidence for miRNA-mediated PTEN modulation in HNSCC cells.

Recent evidence also suggests a powerful association between transforming growth factor-β (TGF-β) signaling and PTEN loss occurs in HNSCC development. The TGF-β superfamily of ligands and receptors represent a signaling pathway unified by a shared group of second messengers: SMADs. While SMAD4 knockdown is sufficient to develop HNSCC in mouse models [120], conditional knockdown of TGF-β receptor 1 (TGF-βRI) in the oral cavity of mice will only lead to early HNSCC development when combined with a topical carcinogen (DMBA) [121]. Consequently, this suggests that progressive disease requires an additional genetic

aberration provided by chemical treatment. Sixteen weeks after DMBA treatment, 45 % of the TGF-βRI conditional knockdown mice develop HNSCC. After 1 year, only 10 % of these mice develop HNSCC without DMBA treatment [122]. TGF-βRI knockdown tumors are characterized by an increase in AKT activity with a paradoxical upregulation of PTEN. However, when PTEN is conditionally knocked-down in combination with TGF-βRI loss, the mice develop benign papillomas within 4 weeks. After 10 weeks, 100 % of the mice develop HNSCC [122]. These tumors demonstrate an overexpression of EGFR and an activation of AKT, NF-κB, and STAT3 signaling, characteristic hallmarks of the human disease. Treatment of these animals with rapamycin effectively prevents tumorigenesis, thus carcinogenesis in this model is an mTOR-dependent event [123]. An overexpression of chemokines and recruitment of tumor-promoting myeloid-derived suppressor cells (CD11b +) is also observed in these carcinomas. Consequently, multiple routes of PI3K/PTEN/AKT/mTOR deregulation contribute to HNSCC initiation, development, and progression.

7.4 Differences in PI3K-Dependent Signaling Based on HPV Status

7.4.1 PI3K Signaling in HPV-negative HNSCC

The most common genetic abnormality associated with human papillomavirus (HPV)-negative HNSCC is a functional loss of p53 [16], [17], yet somatic ablation of this tumor suppressor in transgenic mice favors spontaneous tumor formation in the skin, rather than tumorigenesis in the oral mucosa [124]. However, when p53 loss is combined with constitutively active AKT (myrAKT), tumor formation within the oral cavity, palate, ventral side of the tongue, and lips is markedly increased [125]. These tumors also exhibit increased EGFR expression and potently activated NF-κB and STAT3 pathways, recapitulating the hallmarks of HPV-negative HNSCC. As the PI3K/PTEN/AKT/mTOR pathway can be activated through a multitude of mechanisms, these data suggest that any manner of AKT activation, when combined with p53 loss, may synergize to initiate HNSCC development and progression. This is consistent with the observation that both *PIK3CA* and PTEN deregulation are early events in HNSCC and targeting this pathway may have a role in chemoprevention for smokers.

The majority of patients with HPV-negative tumors have an extensive history of cigarette smoking, and these tumors are associated with an increased number of mutations compared to their HPV-positive cohorts [16], [17]. Tobacco use is a well-defined causal link for the development of HNSCC [126], and cigarette pack-years is a predictive variable for survival even among HPV-positive patients [127]. Studies have shown that nicotine and an additional tobacco carcinogen [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK] are both capable of activating AKT by receptor-mediated signaling in normal human airway epithelial cells [128]. This mechanism

has since been observed in human head and neck epithelium as well, where activated AKT is four-times more likely in HNSCC-adjacent mucosa from smokers compared to nonsmokers [129]. Experiments with HNSCC cell lines have also determined NNK activation of AKT is PI3K dependent [129]. Furthermore, cigarette smoke condensate (CSC) also upregulates the multidrug-transporter ABCG2 in lung cancer and HNSCC-cell lines [130]. After CSC treatment, these cells are more resistant to doxorubicin and have upregulated drug efflux mechanisms; the latter effect can be abrogated by PI3K or nicotinic acetylcholine receptor inhibition [130]. As tobacco use is a strong predictor of HNSCC recurrence, and HNSCC patients have a 10–14 % risk of developing a second malignancy within 5 years of primary surgical treatment [127], [131], further studies of premalignant PI3K/AKT/mTOR activation may yield novel chemopreventive options to mitigate this public health challenge.

Initial studies in recurrence prevention have focused on the therapeutic value of 13-*cis*-retinoic acid (13-cRA). Early data suggested that second primary tumor (SPT) development or HNSCC recurrence may be prevented by 13-cRA treatment [132], [133]. However, in a follow-up phase III clinical trial, no significant difference in SPT or recurrence could be observed between placebo and low-dose 13-cRA in early-stage HNSCC patients [134]. A recent retrospective study characterized 137 SNPs as predictive biomarkers for recurrence in the aforementioned placebo cohort. While 22 SNPs were significantly associated with recurrence, 15 SNPs were detected in the majority of patients who recurred [135]. Ten of these fifteen SNPs were located in TSC1, a negative regulator of mTOR. When these SNPs were assayed in the 13-cRA treatment group, two of the TSC-1-associated SNPs yielded a 43 % decrease in SPT/recurrence with treatment. Variants of *PIK3CD* and *PTEN* were also associated with a decrease in SPT/recurrence risk with 13-cRA treatment [135]. Consequently, prospective analysis of early stage HNSCC for PI3K/PTEN/AKT/mTOR pathway genetic variants could increase the efficacy of 13-cRA chemoprevention.

7.4.2 PI3K Signaling in HPV-positive HNSCC

The incidence of HPV-negative HNSCC is decreasing worldwide following successful tobacco cessation campaigns; however, the overall incidence of HNSCC remains constant due to an increase in HPV-positive HNSCC [136], [137]. From 1988 to 2004, the incidence of HPV-positive HNSCC increased by 225 % in the USA, while the incidence of HPV-negative disease decreased by 50 % over the same time period [137]. HPV-positive HNSCC is primarily associated with tumors of the oropharynx, as HPV infection most commonly occurs in the palatine and lingual tonsils (reviewed in [138]). Mounting evidence suggests PI3K/PTEN/AKT/mTOR signaling has an important role in HPV infection and HPV-induced carcinogenesis.

Recent studies have demonstrated that EGFR and PI3K signaling are required for viral entry into the cell. Pretreatment of HaCaT or cervical cancer cells (HeLa) *in vitro* with an EGFR inhibitor (gefitinib) is sufficient to inhibit HPV-16 endocytosis [139]. Additionally, two different PI3K inhibitors (PI-103, wortmannin) are also

capable of preventing viral entry [139]. As the same mechanism was observed in cells from different anatomical sites, these data suggest EGFR and PI3K activity share a common regulatory requirement for high-risk HPV infection.

However, following viral transformation, the PI3K pathway continues to play an important role in HPV-related tumorigenesis. Gene expression profile analysis of HNSCC patient samples has determined that HPV-positive tumors experience an upregulation in genes associated with the 3q26–29 chromosomal region [140]. This locus contains *PIK3CA*, and confirmatory analysis with RT-PCR confirmed that *PIK3CA* is upregulated in HPV-positive tumors compared to HPV-negative samples [141]. Immunohistochemical (IHC) analysis of HNSCC tissue samples has also demonstrated a strong correlation between p16 upregulation (surrogate marker for HPV-infection) and activated eIF4E ($p = 0.03$) [142]. Although an association between phospho-AKT and p16 expression trended towards significance ($p = 0.06$), its lack of concordance could be caused by additional signaling factors that regulate AKT activity compared to eIF4E function. Consequently, HPV infection is associated with mTOR-dependent activation of mRNA translation, including the upregulation of transformation-related and prosurvival pathway members [143], [144].

In a larger follow-up study, neither phospho-AKT (pAKT S473) nor phospho-S6 (mTOR target) was associated with HPV-positive HNSCCs [145]. In addition, the HPV-positive tumors were not associated with an activation of EGFR, as observed in the HPV-negative samples. Thus, the hyperactivation of selective mTOR targets noted in the prior study may be due to a different mechanism. However, detection of phosphorylated proteins such as AKT and S6 in clinical specimens is challenging due to rapid dephosphorylation and technical variations between studies [146]. To determine if this pathway represents a viable clinical target, HPV-positive HNSCC and cervical cancer xenografts were treated with rapamycin and RAD001 (everolimus). Both xenograft models demonstrated a durable, cytostatic response following mTOR inhibition [145]. Consequently, mTOR inhibition may represent an important therapeutic option in HNSCC patients, particularly those with HPV-positive disease.

7.5 PI3K/AKT/mTOR Inhibition as a Novel Therapeutic Option in HNSCC

Due to the overwhelming preclinical evidence that PI3K/AKT/mTOR signaling represents an integral component of HNSCC signal transduction, a number of clinical trials are currently underway to evaluate the efficacy of small molecules which inhibit key nodes of this pathway (Table 7.2). Currently, rapamycin and its associated analogs (rapalogs) are the most investigated PI3K/AKT/mTOR-targeted agents in HNSCC clinical trials. Rapamycin is a secondary metabolite produced by *Streptomyces hygroscopicus*, isolated from a soil sample collected on Easter Island (Rapa Nui) [147]. Owing to the evolutionarily conserved nature of mTOR, rapamycin exhibits a broad range of antiproliferative activity, and this compound was

Table 7.2 Active clinical trials evaluating PI3K/AKT/mTOR targeted agents for which locally advanced, and recurrent and/or metastatic HNSCC patients are eligible. (All trial data available at www.clinicaltrials.gov)

Targeted agent	Additional targeted agent	Additional therapy	Inclusion criteria	Phase	Clinical trial identifier
mTOR inhibitor					
Everolimus			Advanced HNSCC	II	NCT01111058
			Advanced/recurrent HNSCC	II	NCT01051791
		IMRT, cisplatin	Advanced HNSCC	I	NCT00858663
		Docetaxel, cisplatin	Advanced HNSCC	I	NCT00935961
		Carboplatin, paclitaxel	Advanced HNSCC	I/II	NCT01333085
	Cetuximab	Carboplatin, paclitaxel	Recurrent HNSCC	I/II	NCT01283334
	Cetuximab	Cisplatin, paclitaxel	Advanced HNSCC	II	NCT01133678
	Erlotinib		Recurrent HNSCC	II	NCT00942734
	Vatalinib		Advanced solid tumors	I	NCT00655655
Temsirolimus			Recurrent HNSCC	II	NCT01172769
		Carboplatin, paclitaxel	Advanced/recurrent HNSCC	I/II	NCT01016769
	Cetuximab		Recurrent HNSCC	II	NCT01256385
	Cetuximab	Cisplatin	Advanced/recurrent HNSCC	I/II	NCT01015664
	Erlotinib		Platinum-refractory or ineligible HNSCC	II	NCT01009203
Sirolimus			Advanced HNSCC	I/II	NCT01195922
		Grapefruit juice	Advanced solid tumors	I	NCT00375245
Ridaforolimus	Cetuximab		Advanced HNSCC/NSCLC/CRC	I	NCT01212627
Metformin		Paclitaxel	Recurrent/met HNSCC	II	NCT01333852
PI3K inhibitor					
PX-866		Docetaxel	Advanced HNSCC/NSCLC	I/II	NCT01204099
	Cetuximab		Advanced HNSCC/CRC	I/II	NCT01252628
Dual PI3K/mTOR inhibitor					
NVP-BEZ235			Advanced/met solid tumors	I	NCT01343498
	NVP-BKM120	Paclitaxel	Advanced/met solid tumors	I	NCT01285466
	MEK162		Advanced solid tumors	I	NCT01337765
	Everolimus		Advanced solid tumors	I/II	NCT01508104

HNSCC head and neck squamous cell carcinoma, *IMRT* intensity modulated radiation therapy, *NSCLC* non-small cell lung cancer, *CRC* colorectal cancer

invaluable in elucidating the mechanisms of PI3K/AKT/mTOR signaling. This molecule is an allosteric inhibitor of mTOR, creating a complex with FKBP12 which binds and prevents mTOR activation via the FKBP12-rapamycin-binding (FRB) domain. As this functional domain is unique to mTOR, rapamycin-induced inhibition of mTORC1 is highly selective; however, as reviewed above, mTORC2 is largely uninhibited by this compound in the acute setting [148].

A panel of rapamycin analogs have been synthesized, however intellectual property concerns were not the driving factors in rapalog development. The current array of rapalogs were designed to improve the pharmacokinetics of the parent compound. Temsirolimus (Torisel; Wyeth) is a water-soluble ester of rapamycin (sirolimus) for oral or IV administration (reviewed in [149]). Everolimus (Afinitor; Novartis) is a hydroxyethyl ether derivative, also demonstrating increased solubility relative to the parent compound (reviewed in [150]). Current HNSCC clinical trials are investigating these compounds as single agents or in combination with previously established radiation and chemotherapy regimens. Metformin, while not a direct inhibitor of mTOR, is also being investigated as a chemotherapeutic in this patient setting. Metformin is currently used to control type II diabetes as this compound indirectly inhibits mTORC1 by increasing intracellular AMP levels. Current evidence suggests metformin-induced mTOR inhibition can be mediated by AMPK-dependent and -independent mechanisms [151]–[153]. Additionally, this drug has demonstrated chemopreventive activity for a number of different cancers in diabetic patients (reviewed in [154]). Thus, additional studies are warranted to determine whether this well-characterized compound will have similar chemopreventive or chemotherapeutic effects in non-diabetic patients.

Additional compounds acting upstream of mTOR are also being evaluated in HNSCC. MK-2206 is an allosteric AKT inhibitor developed by Merck. Synergistic anticancer properties have been observed *in vitro* when this compound is used in combination with erlotinib [non-small cell lung cancer, (NSCLC)] or lapatinib (breast cancer) [155]. PX-866 is a synthetic derivative of wortmannin with antineoplastic activity and reduced liver toxicity with respect to the parent compound [156]. Aside from increased safety, PX-866 also demonstrates superior water solubility, bioavailability, and AKT inhibition. However, due to the positive feedback and compensation that can occur via mTORC2, single target inhibition has demonstrated acquired resistance in preclinical and clinical trial investigations. Consequently, additional studies are investigating dual-target inhibitors. NVP-BEZ235 is an orally available, dual PI3K/mTOR inhibitor which reversibly inhibits class I PI3K through ATP competition. This compound is unique because it simultaneously inhibits mTOR catalytic activity while preserving off-target protein kinase function [157], although initial studies suggest the potency of NVP-BEZ235 is not equivalent for each target. In breast cancer cells, NVP-BEZ235 exerts anti-mTOR activity at lower doses (< 100 nM) while dual PI3K/mTOR blockade occurs at higher concentrations (> 500 nM) [158].

7.6 PI3K Pathway Biomarkers

7.6.1 Activation of PI3K Pathway Prognostic Biomarkers

Studies evaluating deregulated PI3K/AKT/mTOR pathway members as prognostic biomarkers in HNSCC are currently ongoing. However, validated data are sparse due to small sample sizes, technical limitations, and the intrinsic biological heterogeneity of HNSCC. To date, the most established prognostic biomarkers for HNSCC outcome are EGFR overexpression and HPV status [127], [159], and the contribution of PI3K/PTEN/AKT/mTOR signaling needs to be evaluated in the context of these well-characterized biomarkers for clinical translation. Initially, investigators have looked at hyperphosphorylation of PI3K/AKT/mTOR pathway members to establish pathway activation. One potential prognostic biomarker is phospho-AKT. Activated AKT is associated with poor local control in HNSCC tumor samples [160]. eIF4E, a downstream target of mTOR, is also upregulated in many HNSCC tumor samples [161]. This protein is also associated with disease recurrence when upregulated eIF4E is detected in tumor-free margins [162].

However, phospho-protein detection in clinical samples can present a challenge as outcomes can differ depending on the fixation protocol and handling time *ex vivo*. Thus, biomarkers utilizing total protein fractions may be preferable from a technical standpoint. One controversy in the literature involves the prognostic value of PTEN in predicting outcome following HNSCC surgery and radiotherapy. In a recent IHC analysis of 140 HNSCC tissue microarray samples, PTEN-positive tumors were associated with worse locoregional control (LRC) than PTEN-negative tumors following surgery and radiation therapy (HR: 2.4) [163]. Additionally, phospho-AKT was also associated with poor LRC (HR: 2.2) and the authors suggest PTEN-positive tumors exhibit increased EGFR activity and subsequent PI3K/AKT/mTOR activation provides a protective effect from ionizing radiation. However, a similar study of 147 HNSCC patients also treated with surgery and radiotherapy observed the opposite association between PTEN and LRC. In this study, the 5-year LRC-free rate for PTEN-low tumors was 52.3 %, while 80.9 % of PTEN-high patients were recurrence free over the same time period ($p = 0.0007$) [164]. Furthermore, PTEN-status did not correlate with 5-year risk of metastasis in this study ($p = 0.49$) [164]. The observed discrepancy between these two studies highlights the difficulty in utilizing a tumor suppressor as a predictive biomarker. In the first study, the authors utilized a 7.5 % tumor cell cutoff to characterize a tumor as PTEN positive or negative by IHC [163]. In the latter study, the intensity of staining was scored by a pathologist, and tumors were described as PTEN high or PTEN low. As PTEN haploinsufficiency can be tumorigenic [28], [29], and techniques for accurate, quantitative assessment of this target from clinical samples are lacking, further studies are required to establish this protein as a bona fide prognostic marker in HNSCC.

On the other hand, detection of *PIK3CA* mutations is more straightforward with current technology. As reviewed above, mutations in exon 9 and 20 are the predominant *PIK3CA* lesions associated with most cancers, and current evidence suggests

these species may contribute differently to clinical outcome. Although important, the lower incidence of these mutations in HNSCC will require large sample sizes to achieve the statistical power needed to delineate any prognostic significance between *PIK3CA* alterations. For example, studies in breast cancer have shown that *PIK3CA* exon 9 mutations (E542/E545) are independently associated with shorter disease-free survival ($p = 0.0003$) and overall survival ($p = 0.001$) [165]. Conversely, exon 20 mutations (H1047) are associated with better overall survival. In this case, the relevance of these biomarkers is intrinsically linked with breast cancer-specific treatments; however, early evidence suggests these mutations need to be evaluated independently rather than in the context of a simplified binomial analysis.

7.6.2 PI3K Pathway Members as Predictive Biomarkers for Radiation Therapy and PI3K/mTOR Inhibitor Response

The association of increased phospho-AKT and poor LRC suggests that AKT activation may be a predictive marker of radiation resistance in HNSCC. While *in vitro* studies have indicated PI3K inhibition increases the radiosensitivity of HNSCC cell lines [160], a recent case report may provide preliminary clinical evidence for an expansion of PI3K/AKT/mTOR combination therapies in HNSCC patients. In this report, the authors describe a patient treated with radiotherapy for squamous cell carcinoma of the larynx (T2N0M0). This patient had also received a liver transplant and was being treated with sirolimus to prevent transplant rejection. After seven fractions of radiation, the patient experienced a complete response, an early response time compared to historical norms. However, treatment-associated toxicities required cessation of radiotherapy [166]. Future investigations of mTOR inhibition as a radiosensitizer in HNSCC treatment may establish an optimal treatment regimen and determine the maximum tolerated dose for this disease.

Aside from radiotherapy, PI3K pathway deregulation may also serve as a biomarker for response to PI3K/AKT/mTOR-targeted agents. A recent retrospective analysis determined the PI3K mutational status of various solid tumors from clinical trials investigating PI3K/AKT/mTOR inhibitors [167]. Of the 1,012 patients in this study, 105 were prospectively selected and 66 of these patients harbored *PIK3CA* mutations. Although these patients had tumors of varying anatomical location, those possessing an exon 20 mutation responded better to PI3K/AKT/mTOR therapies than other PI3K mutants (PR rate: 38 vs. 10 %, $p = 0.018$). Unfortunately, an increase in progression-free survival only trended towards statistical significance (5.7 vs. 2 months, $p = 0.06$). While this study was hampered by a heterogeneous tumor population and multiple treatment regimens, PI3K/AKT/mTOR-treatment efficacy in patients with exon 20 mutations is an intriguing finding that requires further study. Of the 66 prospectively selected patients from this study, four individuals had HNSCC and the best responder possessed an H1047R mutation. Although these data indicate PI3K mutations may sensitize HNSCC tumors to PI3K/AKT/mTOR inhibitor treatment, wild-type PI3K status may not preclude the use of these drugs in this

patient population. While HNSCC cell lines have demonstrated *in vitro* sensitivity to PI3K inhibition, the efficacy of this treatment option is enhanced when combined with vorinostat, a histone deacetylase (HDAC) inhibitor [168]. This combination treatment is capable of increasing reactive oxygen species (ROS) production in a manner previously observed with other efficacious cytotoxic chemotherapeutics [169], and is only toxic to HNSCC cell lines, not keratinocytes [168].

7.6.3 Predictive Biomarkers for Receptor Tyrosine Kinase (RTK) Inhibitor Resistance

While the identification of predictive biomarkers for RTK inhibitor response is of paramount concern, equally important is the investigation of biomarkers for resistance. For example, additional HER receptors, aside from EGFR, signal through PI3K in HNSCC. A positive feedback loop has been reported between HER2 and ADAM12 in HNSCC cell lines. ADAM12 is a multifunctional protein with an intracellular domain capable of second messenger signaling and an extracellular domain capable of cleaving extracellular matrix substrates and activating EGFR ligands [170]–[172]. HER2 and ADAM12 have the ability to upregulate each other in HNSCC cell lines, and this positive feedback loop is dependent on PI3K and JNK signaling [173]. Additionally, ADAM12 upregulation confers increased migratory and invasive phenotypes to these cells. This signaling mechanism may have clinical significance as HER2 activation and total HER3 expression are predictive of *de novo* resistance to gefitinib (EGFR-targeted TKI) [174]. While upregulated ligand converting enzymes can potentially serve as biomarkers of therapy resistance, their cleavage products can also subvert targeted therapeutic response. For example, an upregulation of heparin-binding EGF (HB-EGF) has been observed in HNSCC cell lines with acquired resistance to cetuximab [175]. Increased serum HB-EGF plasma levels are also detected in patients with recurrent disease compared to those who are newly diagnosed.

A strong relationship also exists between Met and PI3K signaling in HNSCC. Consequently, Met activation represents another potential source of PI3K-mediated RTK inhibitor resistance. To address this concern, potent Met inhibitors (SU11274 and PF-2341066) have been developed and pretreatment of HNSCC cell lines with these compounds *in vitro* does prevent ligand-induced AKT activation [176], [177]. However, the degree of concordance between AKT inhibition and pharmacologic Met inhibition depends on which AKT phosphorylation site is studied. One investigation demonstrated consistent AKT inhibition with Met inhibitors when utilizing the mTORC2 phosphorylation site (S473) as a readout of AKT activity [176]. Meanwhile a similar investigation observed modest AKT inhibition across a panel of HNSCC cell lines while employing the PDK-1 phosphorylation site (T308) as a marker [177]. Due to the differential regulation of these sites, it is quite possible both observations are valid and these parallel studies provide further insight into the PI3K/PTEN/AKT/mTOR signaling occurring downstream of Met. From a clinical

perspective, the dual regulation of AKT may explain why combined treatments of EGFR and Met TKIs have potent, additive effects on HNSCC growth inhibition [177], [178]. Met activation following the addition of EGFR ligands has also been observed, suggesting crosstalk between these two receptors may have an important functional role [178]. As discussed above, while multiple pathways interact with Met, current evidence suggests PI3K/AKT/mTOR signaling is specifically capable of mediating pathologic signal transduction downstream of this receptor.

An additional pathway providing inhibitor resistance during HNSCC treatment is the TGF-β pathway. Aside from ligand-mediated signaling, noncanonical TGF-β activation can occur through the downstream pathways shared with EGFR and Met (MAPK, PI3K/AKT, and Rho GTPase) [179]. However, recent evidence suggests that TGF-β-induced changes in the tumor microenvironment can inhibit ADCC while simultaneously activating tumor-associated AKT signaling [180]. In this paradigm, TGF-β1 reduces the efficacy of immune-associated responses to cetuximab treatment while concurrently providing a proliferative signal to the tumor. In support of this hypothesis, HNSCC xenografts selected *in vivo* for cetuximab-resistance display increased TGF-β expression and TGF-β-dependent AKT activation [180]. This resistance is reversible with a TGF-β inhibitor, providing strong preclinical evidence for this therapeutic option in cetuximab-refractory HNSCCs.

7.7 Conclusion

Overwhelming evidence suggests the PI3K/PTEN/AKT/mTOR pathway is commonly deregulated in HNSCC. Although excellent preclinical and clinical studies have begun evaluating the therapeutic potential of this pathway in HNSCC, additional work is required to verify nodes of oncogenic dependency and addiction in this signaling network. Once these targets are fully validated in the laboratory, we can translate these findings to the clinic and identify the appropriate population of need. While single agent therapies targeting this pathway may represent future clinical endeavors, these compounds may also serve to enhance the efficacy of standard therapy options in use today. Whether these targeted agents will prove efficacious in HNSCC treatment is not clear, but the role of PI3K/PTEN/AKT/mTOR pathway deregulation in HNSCC certainly warrants further investigation.

References

1. Pedrero JM, Carracedo DG, Pinto CM, Zapatero AH, Rodrigo JP, Nieto CS, Gonzalez MV. Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. Int J Cancer. 2005;114(2):242–8. doi:10.1002/ijc.20711.
2. Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, et al. PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO J. 1994;13(3):522–33.
3. Foukas LC, Beeton CA, Jensen J, Phillips WA, Shepherd PR. Regulation of phosphoinositide 3-kinase by its intrinsic serine kinase activity in vivo. Mol Cell Biol. 2004;24(3):966–75.

4. Kang S, Denley A, Vanhaesebroeck B, Vogt PK. Oncogenic transformation induced by the p110beta, -gamma, and -delta isoforms of class I phosphoinositide 3-kinase. Proc Natl Acad Sci U S A. 2006;103(5):1289–94. doi:0510772103 [pii]. 10.1073/pnas.0510772103.
5. Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell. 1991;65(1):83–90. doi:0092-8674(91)90410-Z [pii].
6. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell. 1994;77(1):83–93. doi:0092-8674(94)90237-2 [pii].
7. Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature. 1994;370(6490):527–32. doi:10.1038/370527a0.
8. Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD, Downward J. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. EMBO J. 1996;15(10):2442–51.
9. Chan TO, Rodeck U, Chan AM, Kimmelman AC, Rittenhouse SE, Panayotou G, Tsichlis PN. Small GTPases and tyrosine kinases coregulate a molecular switch in the phosphoinositide 3-kinase regulatory subunit. Cancer Cell. 2002;1(2):181–91. doi:S1535610802000338 [pii].
10. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 1997;7(4):261–9. doi:S0960-9822(06)00122-9 [pii].
11. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1998;273(22):13375–8.
12. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell. 1998;95(1):29–39. doi:S0092-8674(00)81780-8 [pii].
13. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. High frequency of mutations of the PIK3CA gene in human cancers. Science. 2004;304(5670):554. doi:10.1126/science.1096502. 1096502 [pii].
14. Qiu W, Schonleben F, Li X, Ho DJ, Close LG, Manolidis S, Bennett BP, Su GH. PIK3CA mutations in head and neck squamous cell carcinoma. Clin Cancer Res. 2006;12(5):1441–6. doi:12/5/1441 [pii]. 10.1158/1078-0432.CCR-05-2173.
15. Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N. Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. Int J Oncol. 2008;32(1):101–11.
16. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60. doi:science.1208130 [pii]. 10.1126/science.1208130.
17. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs RA, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–7. doi:science.1206923 [pii]. 10.1126/science.1206923.

18. Huang CH, Mandelker D, Schmidt-Kittler O, Samuels Y, Velculescu VE, Kinzler KW, Vogelstein B, Gabelli SB, Amzel LM. The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. Science. 2007;318(5857):1744–8. doi:318/5857/1744 [pii]. 10.1126/science.1150799.
19. Miled N, Yan Y, Hon WC, Perisic O, Zvelebil M, Inbar Y, Schneidman-Duhovny D, Wolfson HJ, Backer JM, Williams RL. Mechanism of two classes of cancer mutations in the phosphoinositide 3-kinase catalytic subunit. Science. 2007;317(5835):239–42. doi:317/5835/239 [pii]. 10.1126/science.1135394.
20. Zhao L, Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. Proc Natl Acad Sci U S A. 2008;105(7):2652–7. doi:0712169105 [pii]. 10.1073/pnas.0712169105.
21. Ikenoue T, Kanai F, Hikiba Y, Obata T, Tanaka Y, Imamura J, Ohta M, Jazag A, Guleng B, Tateishi K, Asaoka Y, Matsumura M, Kawabe T, Omata M. Functional analysis of PIK3CA gene mutations in human colorectal cancer. Cancer Res. 2005;65(11):4562–7. doi:65/11/4562 [pii]. 10.1158/0008-5472.CAN-04-4114.
22. Gymnopoulos M, Elsliger MA, Vogt PK. Rare cancer-specific mutations in PIK3CA show gain of function. Proc Natl Acad Sci U S A. 2007;104(13):5569–74. doi:0701005104 [pii] 10.1073/pnas.0701005104.
23. Bigner SH, Mark J, Mahaley MS, Bigner DD. Patterns of the early, gross chromosomal changes in malignant human gliomas. Hereditas. 1984;101(1):103–13.
24. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275(5308):1943–7.
25. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet. 1997;15(4):356–62. doi:10.1038/ng0497-356.
26. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet. 1997;16(1):64–7. doi:10.1038/ng0597-64.
27. Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A. 1999;96(4):1563–8.
28. Di Cristofano A, Kotsi P, Peng YF, Cordon-Cardo C, Elkon KB, Pandolfi PP. Impaired Fas response and autoimmunity in Pten+/− mice. Science. 1999;285(5436):2122–5. doi:7853 [pii].
29. Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP. Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. Nat Genet. 2001;27(2):222–4. doi:10.1038/84879.
30. Martin-Belmonte F, Gassama A, Datta A, Yu W, Rescher U, Gerke V, Mostov K. PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. Cell. 2007;128(2):383–97. doi:S0092-8674(07)00005-0 [pii].10.1016/j.cell.2006.11.051.
31. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell. 2008;14(6):818–29. doi:S1534-5807(08)00209-8 [pii]. 10.1016/j.devcel.2008.05.009.
32. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, Yin Y. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007;128(1):157–70. doi:S0092-8674(06)01553-4 [pii]. 10.1016/j.cell.2006.11.042.
33. Lindsay Y, McCoull D, Davidson L, Leslie NR, Fairservice A, Gray A, Lucocq J, Downes CP. Localization of agonist-sensitive PtdIns(3,4,5)P3 reveals a nuclear pool that is insensitive to PTEN expression. J Cell Sci. 2006;119(Pt 24):5160–8. doi:119/24/5160 [pii]. 10.1242/jcs.000133.

34. Staal SP, Hartley JW, Rowe WP. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. Proc Natl Acad Sci U S A. 1977;74(7): 3065–7.
35. Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science. 1991;254(5029):274–7.
36. Coffer PJ, Woodgett JR. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur J Biochem. 1991;201(2):475–81.
37. Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A. 1991;88(10):4171–5.
38. Bellacosa A, Franke TF, Gonzalez-Portal ME, Datta K, Taguchi T, Gardner J, Cheng JQ, Testa JR, Tsichlis PN. Structure, expression and chromosomal mapping of c-akt: relationship to v-akt and its implications. Oncogene. 1993;8(3):745–54.
39. Jones PF, Jakubowicz T, Hemmings BA. Molecular cloning of a second form of rac protein kinase. Cell Regul. 1991;2(12):1001–9.
40. Konishi H, Shinomura T, Kuroda S, Ono Y, Kikkawa U. Molecular cloning of rat RAC protein kinase alpha and beta and their association with protein kinase C zeta. Biochem Biophys Res Commun. 1994;205(1):817–25. doi:S0006-291X(84)72738-0 [pii]. 10.1006/bbrc.1994.2738.
41. Nakatani K, Sakaue H, Thompson DA, Weigel RJ, Roth RA. Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site. Biochem Biophys Res Commun. 1999;257(3):906–10. doi:S0006-291X(99)90559-4 [pii]. 10.1006/bbrc.1999.0559.
42. Sjoblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. Science. 2006;314(5797):268–74. doi:1133427 [pii]. 10.1126/science.1133427.
43. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR. Patterns of somatic mutation in human cancer genomes. Nature. 2007;446(7132):153–8. doi:nature05610 [pii]. 10.1038/nature05610.
44. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science. 2001;292(5522):1728–31. doi:10.1126/science.292.5522.1728. 292/5522/1728 [pii].
45. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem. 2001;276(42):38349–52. doi:10.1074/jbc.C100462200. C100462200 [pii].
46. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. 2001;15(17):2203–8. doi:10.1101/gad.913901.
47. Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM, Szabolcs M, de Jong R, Oltersdorf T, Ludwig T, Efstratiadis A, Birnbaum MJ. Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Mol Cell Biol. 2005;25(5):1869–78. doi:25/5/1869 [pii]. 10.1128/MCB.25.5.1869-1878.2005.

48. Tschopp O, Yang ZZ, Brodbeck D, Dummler BA, Hemmings-Mieszczak M, Watanabe T, Michaelis T, Frahm J, Hemmings BA. Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. Development. 2005;132(13):2943–54. doi:dev.01864 [pii]. 10.1242/dev.01864.
49. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science. 1997;275(5300):665–8.
50. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005;307(5712):1098–101. doi:307/5712/1098 [pii]. 10.1126/science.1106148.
51. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 1996;15(23):6541–51.
52. Mahajan K, Coppola D, Challa S, Fang B, Chen YA, Zhu W, Lopez AS, Koomen J, Engelman RW, Rivera C, Muraoka-Cook RS, Cheng JQ, Schonbrunn E, Sebti SM, Earp HS, Mahajan NP. Ack1 mediated AKT/PKB tyrosine 176 phosphorylation regulates its activation. PLoS One. 2010;5(3):e9646. doi:10.1371/journal.pone.0009646.
53. Mahajan K, Mahajan NP. Shepherding AKT and androgen receptor by Ack1 tyrosine kinase. J Cell Physiol. 2010;224(2):327–33. doi:10.1002/jcp.22162.
54. Chen R, Kim O, Yang J, Sato K, Eisenmann KM, McCarthy J, Chen H, Qiu Y. Regulation of Akt/PKB activation by tyrosine phosphorylation. J Biol Chem. 2001;276(34):31858–62. doi:10.1074/jbc.C100271200. C100271200 [pii].
55. Zheng Y, Peng M, Wang Z, Asara JM, Tyner AL. Protein tyrosine kinase 6 directly phosphorylates AKT and promotes AKT activation in response to epidermal growth factor. Mol Cell Biol. 2010;30(17):4280–92. doi:MCB.00024-10 [pii]. 10.1128/MCB.00024-10.
56. Joung SM, Park ZY, Rani S, Takeuchi O, Akira S, Lee JY. Akt contributes to activation of the TRIF-dependent signaling pathways of TLRs by interacting with TANK-binding kinase 1. J Immunol. 2011;186(1):499–507. doi:jimmunol.0903534 [pii]. 10.4049/jimmunol.0903534.
57. Ou YH, Torres M, Ram R, Formstecher E, Roland C, Cheng T, Brekken R, Wurz R, Tasker A, Polverino T, Tan SL, White MA. TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell. 2011;41(4):458–70. doi:S1097-2765(11)00046-3 [pii]. 10.1016/j.molcel.2011.01.019.
58. Xie X, Zhang D, Zhao B, Lu MK, You M, Condorelli G, Wang CY, Guan KL. IkappaB kinase epsilon and TANK-binding kinase 1 activate AKT by direct phosphorylation. Proc Natl Acad Sci U S A. 2011;108(16):6474–9. doi:1016132108 [pii]. 10.1073/pnas.1016132108.
59. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A. 2001;98(20):11598–603. doi:10.1073/pnas.181181198. 181181198.
60. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol. 2001;3(3):245–52. doi:10.1038/35060032.
61. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 1998;12(22):3499–511.
62. Wei W, Jin J, Schlisio S, Harper JW, Kaelin WG Jr. The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell. 2005;8(1):25–33. doi:S1535-6108(05)00193-5 [pii]. 10.1016/j.ccr.2005.06.005.
63. Welcker M, Singer J, Loeb KR, Grim J, Bloecher A, Gurien-West M, Clurman BE, Roberts JM. Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. Mol Cell. 2003;12(2):381–92. doi:S1097276503002879 [pii].
64. Yeh E, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat Cell Biol. 2004;6(4):308–18. doi:10.1038/ncb1110. ncb1110 [pii].
65. Huang WC, Chen CC. Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. Mol Cell Biol. 2005;25(15):6592–602. doi:25/15/6592 [pii]. 10.1128/MCB.25.15.6592-6602.2005.

66. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999;96(6):857–68. doi:S0092-8674(00)80595-4 [pii].
67. Laine J, Kunstle G, Obata T, Sha M, Noguchi M. The protooncogene TCL1 is an Akt kinase coactivator. Mol Cell. 2000;6(2):395–407. doi:S1097-2765(00)00039-3 [pii].
68. Pekarsky Y, Koval A, Hallas C, Bichi R, Tresini M, Malstrom S, Russo G, Tsichlis P, Croce CM. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc Natl Acad Sci U S A. 2000;97(7):3028–33. doi:10.1073/pnas.040557697. 040557697 [pii].
69. Cappellini A, Tabellini G, Zweyer M, Bortul R, Tazzari PL, Billi AM, Fala F, Cocco L, Martelli AM. The phosphoinositide 3-kinase/Akt pathway regulates cell cycle progression of HL60 human leukemia cells through cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27(Kip1) and control of cyclin D1 expression. Leukemia. 2003;17(11):2157–67. doi:10.1038/sj.leu.2403111. 2403111 [pii].
70. Brandts CH, Sargin B, Rode M, Biermann C, Lindtner B, Schwable J, Buerger H, Muller-Tidow C, Choudhary C, McMahon M, Berdel WE, Serve H. Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res. 2005;65(21):9643–50. doi:65/21/9643 [pii]. 10.1158/0008-5472.CAN-05-0422.
71. Lee SH, Kim HS, Park WS, Kim SY, Lee KY, Kim SH, Lee JY, Yoo NJ. Non-small cell lung cancers frequently express phosphorylated Akt; an immunohistochemical study. APMIS. 2002;110(7–8):587–92. doi:apm11007-811 [pii].
72. Nicholson KM, Streuli CH, Anderson NG. Autocrine signalling through erbB receptors promotes constitutive activation of protein kinase B/Akt in breast cancer cell lines. Breast Cancer Res Treat. 2003;81(2):117–28. doi:10.1023/A:1025765215765.
73. Vasko V, Saji M, Hardy E, Kruhlak M, Larin A, Savchenko V, Miyakawa M, Isozaki O, Murakami H, Tsushima T, Burman KD, De Micco C, Ringel MD. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. J Med Genet. 2004;41(3):161–70.
74. Van deST, Roskams T, Lerut E, Joniau S, Van Poppel H, Verhoeven G, Swinnen JV. High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. J Pathol. 2005;206(2):214–9. doi:10.1002/path.1760.
75. Montironi R, Mazzuccheli R, Scarpelli M, Lopez-Beltran A, Fellegara G, Algaba F. Gleason grading of prostate cancer in needle biopsies or radical prostatectomy specimens: contemporary approach, current clinical significance and sources of pathology discrepancies. BJU Int. 2005;95(8):1146–52. doi:BJU5540 [pii]. 10.1111/j.1464-410X.2005.05540.x.
76. Trotman LC, Alimonti A, Scaglioni PP, Koutcher JA, Cordon-Cardo C, Pandolfi PP. Identification of a tumour suppressor network opposing nuclear Akt function. Nature. 2006;441 (7092):523–7. doi:nature04809 [pii]. 10.1038/nature04809.
77. Lindhurst MJ, Sapp JC, Teer JK, Johnston JJ, Finn EM, Peters K, Turner J, Cannons JL, Bick D, Blakemore L, Blumhorst C, Brockmann K, Calder P, Cherman N, Deardorff MA, Everman DB, Golas G, Greenstein RM, Kato BM, Keppler-Noreuil KM, Kuznetsov SA, Miyamoto RT, Newman K, Ng D, O'Brien K, Rothenberg S, Schwartzentruber DJ, Singhal V, Tirabosco R, Upton J, Wientroub S, Zackai EH, Hoag K, Whitewood-Neal T, Robey PG, Schwartzberg PL, Darling TN, Tosi LL, Mullikin JC, Biesecker LG. A mosaic activating mutation in AKT1 associated with the Proteus syndrome. N Engl J Med. 2011;365(7):611–9. doi:10.1056/NEJMoa1104017.
78. Biesecker LG. The multifaceted challenges of Proteus syndrome. JAMA. 2001;285(17):2240–3. doi:jgn10000 [pii].
79. Biesecker L. The challenges of Proteus syndrome: diagnosis and management. Eur J Hum Genet. 2006;14(11):1151–7. doi:5201638 [pii]. 10.1038/sj.ejhg.5201638.
80. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, Hostetter G, Boguslawski S, Moses TY, Savage S, Uhlik M, Lin A, Du J, Qian YW, Zeckner DJ, Tucker-Kellogg G, Touchman J, Patel K, Mousses S, Bittner M, Schevitz R, Lai MH, Blanchard KL, Thomas JE. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature. 2007;448(7152):439–44. doi:nature05933 [pii]. 10.1038/nature05933.

81. Askham JM, Platt F, Chambers PA, Snowden H, Taylor CF, Knowles MA. AKT1 mutations in bladder cancer: identification of a novel oncogenic mutation that can co-operate with E17K. Oncogene. 2010;29(1):150–5. doi:onc2009315 [pii]. 10.1038/onc.2009.315.
82. Cohen Y, Shalmon B, Korach J, Barshack I, Fridman E, Rechavi G. AKT1 pleckstrin homology domain E17K activating mutation in endometrial carcinoma. Gynecol Oncol. 2010;116 (1):88–91. doi:S0090-8258(09)00737-9 [pii]. 10.1016/j.ygyno.2009.09.038.
83. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110(2):177–89. doi:S0092867402008334 [pii].
84. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002;110(2):163–75. doi:S0092867402008085 [pii].
85. Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, Hara K, Tanaka N, Avruch J, Yonezawa K. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J Biol Chem. 2003;278(18):15461–4. doi:10.1074/jbc.C200665200. C200665200 [pii].
86. Schalm SS, Fingar DC, Sabatini DM, Blenis J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Curr Biol. 2003;13(10):797–806. doi:S0960982203003294 [pii].
87. Wullschleger S, Loewith R, Oppliger W, Hall MN. Molecular organization of target of rapamycin complex 2. J Biol Chem. 2005;280(35):30697–704. doi:M505553200 [pii]. 10.1074/jbc.M505553200.
88. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008;320(5882):1496–501. doi:1157535 [pii]. 10.1126/science.1157535.
89. Pearce LR, Huang X, Boudeau J, Pawlowski R, Wullschleger S, Deak M, Ibrahim AF, Gourlay R, Magnuson MA, Alessi DR. Identification of Protor as a novel Rictor-binding component of mTOR complex-2. Biochem J. 2007;405(3):513–22. doi:BJ20070540 [pii]. 10.1042/BJ20070540.
90. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell. 2007;25(6):903–15. doi:S1097-2765(07)00148-7 [pii]. 10.1016/j.molcel.2007.03.003.
91. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol. 2007;9(3):316–23. doi:ncb1547 [pii]. 10.1038/ncb1547.
92. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14(14):1296–302. doi:10.1016/j.cub.2004.06.054 S0960982204004713 [pii].
93. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell. 2002;10(3):457–68. doi:S1097276502006366 [pii].
94. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004;6(11):1122–8. doi:ncb1183 [pii]. 10.1038/ncb1183.
95. Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I, Avruch J. Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem. 1997;272(42):26457–63.
96. Ma XM, Yoon SO, Richardson CJ, Julich K, Blenis J. SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. Cell. 2008; 133(2):303–13. doi:S0092-8674(08)00282-1 [pii]. 10.1016/j.cell.2008.02.031.
97. Holz MK, Ballif BA, Gygi SP, Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell. 2005;123(4):569–80. doi:S0092-8674(05)01157-8 [pii]. 10.1016/j.cell.2005.10.024.

98. Mayer C, Zhao J, Yuan X, Grummt I. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev. 2004;18(4):423–34. doi:10.1101/gad.285504. 18/4/423 [pii].
99. Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem. 1998;273(7):3963–6.
100. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, Reichling LJ, Sim T, Sabatini DM, Gray NS. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem. 2009;284(12):8023–32. doi:M900301200 [pii]. 10.1074/jbc.M900301200.
101. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol. 2000;150(6):1507–13.
102. Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA, Sabatini DM. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol. 2006;16(18):1865–70. doi:S0960-9822(06)01974-9 [pii]. 10.1016/j.cub.2006.08.001.
103. Yang Q, Inoki K, Ikenoue T, Guan KL. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev. 2006;20(20):2820–32. doi:20/20/2820 [pii]. 10.1101/gad.1461206.
104. Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ, Sessa WC, Qin J, Zhang P, Su B, Jacinto E. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J. 2008;27(14):1932–43. doi:10.1038/emboj.2008.120. emboj2008120 [pii].
105. Garcia-Martinez JM, Alessi DR. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J. 2008;416(3):375–85. doi:BJ20081668 [pii]. 10.1042/BJ20081668.
106. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J. 2008;27(14):1919–31. doi:emboj2008119 [pii]. 10.1038/emboj.2008.119.
107. van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van denOA, Halley D, Young J, Burley M, Jeremiah S, Woodward K, Nahmias J, Fox M, Ekong R, Osborne J, Wolfe J, Povey S, Snell RG, Cheadle JP, Jones AC, Tachataki M, Ravine D, Sampson JR, Reeve MP, Richardson P, Wilmer F, Munro C, Hawkins TL, Sepp T, Ali JB, Ward S, Green AJ, Yates JR, Kwiatkowska J, Henske EP, Short MP, Haines JH, Jozwiak S, Kwiatkowski DJ. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science. 1997;277(5327):805–8.
108. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. 2003;112(12):1809–20. doi:10.1172/JCI20039. JCI200320039 [pii].
109. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A. 2003;100(25):15077–82. doi:10.1073/pnas.2436255100. 2436255100 [pii].
110. Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem. 2007;282(25):18573–83. doi:M701194200 [pii]. 10.1074/jbc.M701194200.
111. Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, Mullholland DJ, Magnuson MA, Wu H, Sabatini DM. mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. Cancer Cell. 2009;15(2):148–59. doi:S1535-6108(08)00436-4 [pii]. 10.1016/j.ccr.2008.12.017.
112. Hietakangas V, Cohen SM. TOR complex 2 is needed for cell cycle progression and anchorage-independent growth of MCF7 and PC3 tumor cells. BMC Cancer. 2008;8:282. doi:1471-2407-8-282 [pii]. 10.1186/1471-2407-8-282.

113. Woenckhaus J, Steger K, Werner E, Fenic I, Gamerdinger U, Dreyer T, Stahl U. Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma. J Pathol. 2002;198(3):335–42. doi:10.1002/path.1207.
114. Suda T, Hama T, Kondo S, Yuza Y, Yosikawa M, Urashima M, Kato T, Moriyama H. Copy number amplification of the PIK3CA gene is associated with poor prognosis in non-lymph node metastatic head and neck squamous cell carcinoma. BMC Cancer. 2012;12(1):416. doi:1471-2407-12-416 [pii]. 10.1186/1471-2407-12-416.
115. Tsui IF, Poh CF, Garnis C, Rosin MP, Zhang L, Lam WL. Multiple pathways in the FGF signaling network are frequently deregulated by gene amplification in oral dysplasias. Int J Cancer. 2009;125(9):2219–28. doi:10.1002/ijc.24611.
116. Shao X, Tandon R, Samara G, Kanki H, Yano H, Close LG, Parsons R, Sato T. Mutational analysis of the PTEN gene in head and neck squamous cell carcinoma. Int J Cancer. 1998;77(5):684–688. doi:10.1002/(SICI)1097-0215(19980831)77:5 < 684::AID-IJC4 > 3.0.CO;2-R [pii].
117. Lee JI, Soria JC, Hassan KA, El-Naggar AK, Tang X, Liu DD, Hong WK, Mao L. Loss of PTEN expression as a prognostic marker for tongue cancer. Arch Otolaryngol Head Neck Surg. 2001;127(12):1441–5. doi:ooa10112 [pii].
118. Darido C, Georgy SR, Wilanowski T, Dworkin S, Auden A, Zhao Q, Rank G, Srivastava S, Finlay MJ, Papenfuss AT, Pandolfi PP, Pearson RB, Jane SM. Targeting of the tumor suppressor GRHL3 by a miR-21-dependent proto-oncogenic network results in PTEN loss and tumorigenesis. Cancer Cell. 2011;20(5):635–48. doi:S1535-6108(11)00397-7 [pii]. 10.1016/j.ccr.2011.10.014.
119. Minor J, Wang X, Zhang F, Song J, Jimeno A, Wang XJ, Lu X, Gross N, Kulesz-Martin M, Wang D, Lu SL. Methylation of microRNA-9 is a specific and sensitive biomarker for oral and oropharyngeal squamous cell carcinomas. Oral Oncol. 2012;48(1):73–8. doi:S1368-8375(11)00885-2 [pii]. 10.1016/j.oraloncology.2011.11.006.
120. Bornstein S, White R, Malkoski S, Oka M, Han G, Cleaver T, Reh D, Andersen P, Gross N, Olson S, Deng C, Lu SL, Wang XJ. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. J Clin Invest. 2009;119(11):3408–19. doi:10.1172/JCI38854 38854 [pii].
121. Bian Y, Terse A, Du J, Hall B, Molinolo A, Zhang P, Chen W, Flanders KC, Gutkind JS, Wakefield LM, Kulkarni AB. Progressive tumor formation in mice with conditional deletion of TGF-beta signaling in head and neck epithelia is associated with activation of the PI3K/Akt pathway. Cancer Res. 2009;69(14):5918–26. doi:0008-5472.CAN-08-4623 [pii]. 10.1158/0008-5472.CAN-08-4623.
122. Bian Y, Hall B, Sun ZJ, Molinolo A, Chen W, Gutkind JS, Waes CV, Kulkarni AB. Loss of TGF-beta signaling and PTEN promotes head and neck squamous cell carcinoma through cellular senescence evasion and cancer-related inflammation. Oncogene. 2011. doi:10.1038/onc.2011.494. onc2011494 [pii].
123. Sun ZJ, Zhang L, Hall B, Bian Y, Gutkind JS, Kulkarni AB. Chemopreventive and chemotherapeutic actions of mTOR inhibitor in genetically defined head and neck squamous cell carcinoma mouse model. Clin Cancer Res. 2012;18(19):5304–13. doi:1078-0432.CCR-12-1371 [pii]. 10.1158/1078-0432.CCR-12-1371.
124. Martinez-Cruz AB, Santos M, Lara MF, Segrelles C, Ruiz S, Moral M, Lorz C, Garcia-Escudero R, Paramio JM. Spontaneous squamous cell carcinoma induced by the somatic inactivation of retinoblastoma and Trp53 tumor suppressors. Cancer Res. 2008;68(3):683–92. doi:68/3/683 [pii]. 10.1158/0008-5472.CAN-07-3049.
125. Moral M, Segrelles C, Lara MF, Martinez-Cruz AB, Lorz C, Santos M, Garcia-Escudero R, Lu J, Kiguchi K, Buitrago A, Costa C, Saiz C, Rodriguez-Peralto JL, Martinez-Tello FJ, Rodriguez-Pinilla M, Sanchez-Cespedes M, Garin M, Grande T, Bravo A, DiGiovanni J, Paramio JM. Akt activation synergizes with Trp53 loss in oral epithelium to produce a novel mouse model for head and neck squamous cell carcinoma. Cancer Res. 2009;69(3):1099–108. doi:0008-5472.CAN-08-3240 [pii]. 10.1158/0008-5472.CAN-08-3240.

126. Vineis P, Alavanja M, Buffler P, Fontham E, Franceschi S, Gao YT, Gupta PC, Hackshaw A, Matos E, Samet J, Sitas F, Smith J, Stayner L, Straif K, Thun MJ, Wichmann HE, Wu AH, Zaridze D, Peto R, Doll R. Tobacco and cancer: recent epidemiological evidence. J Natl Cancer Inst. 2004;96(2):99–106.
127. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24–35. doi:NEJMoa0912217 [pii]. 10.1056/NEJMoa0912217.
128. West KA, Brognard J, Clark AS, Linnoila IR, Yang X, Swain SM, Harris C, Belinsky S, Dennis PA. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. J Clin Invest. 2003;111(1):81–90. doi:10.1172/JCI16147.
129. Weber SM, Bornstein S, Li Y, Malkoski SP, Wang D, Rustgi AK, Kulesz-Martin MF, Wang XJ, Lu SL. Tobacco-specific carcinogen nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induces AKT activation in head and neck epithelia. Int J Oncol. 2011;39(5):1193–8. doi:10.3892/ijo.2011.1149.
130. An Y, Kiang A, Lopez JP, Kuo SZ, Yu MA, Abhold EL, Chen JS, Wang-Rodriguez J, Ongkeko WM. Cigarette smoke promotes drug resistance and expansion of cancer stem cell-like side population. PLoS One. 2012;7(11):e47919. doi:10.1371/journal.pone.0047919. PONE-D-11-06991 [pii].
131. Lin K, Patel SG, Chu PY, Matsuo JM, Singh B, Wong RJ, Kraus DH, Shaha AR, Shah JP, Boyle JO. Second primary malignancy of the aerodigestive tract in patients treated for cancer of the oral cavity and larynx. Head Neck. 2005;27(12):1042–8. doi:10.1002/hed.20272.
132. Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. N Engl J Med. 1990;323(12):795–801. doi:10.1056/NEJM199009203231205.
133. Benner SE, Pajak TF, Lippman SM, Earley C, Hong WK. Prevention of second primary tumors with isotretinoin in patients with squamous cell carcinoma of the head and neck: long-term follow-up. J Natl Cancer Inst. 1994;86(2):140–1.
134. Khuri FR, Lee JJ, Lippman SM, Kim ES, Cooper JS, Benner SE, Winn R, Pajak TF, Williams B, Shenouda G, Hodson I, Fu K, Shin DM, Vokes EE, Feng L, Goepfert H, Hong WK. Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients. J Natl Cancer Inst. 2006;98(7):441–50. doi:98/7/441 [pii]. 10.1093/jnci/djj091.
135. Hildebrandt MA, Lippman SM, Etzel CJ, Kim E, Lee JJ, Khuri FR, Spitz MR, Lotan R, Hong WK, Wu X. Genetic variants in the PI3K/PTEN/AKT/mTOR pathway predict head and neck cancer patient second primary tumor/recurrence risk and response to retinoid chemoprevention. Clin Cancer Res. 2012;18(13):3705–13. doi:10.1158/1078-0432.CCR-11-3271. 1078-0432.CCR-11-3271 [pii].
136. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol. 2008;26(4):612–9. doi:26/4/612 [pii]. 10.1200/JCO.2007.14.1713.
137. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29(32):4294–301. doi:10.1200/JCO.2011.36.4596. JCO.2011.36.4596 [pii].
138. Howard JD, Chung CH. Biology of human papillomavirus-related oropharyngeal cancer. Semin Radiat Oncol. 2012;22(3):187–93. doi:10.1016/j.semradonc.2012.03.002. S1053-4296(12)00021-5 [pii].
139. Schelhaas M, Shah B, Holzer M, Blattmann P, Kuhling L, Day PM, Schiller JT, Helenius A. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS Pathog. 2012;8(4):e1002657. doi:10.1371/journal.ppat.1002657. PPATHOGENS-D-11-02158 [pii].

140. Slebos RJ, Yi Y, Ely K, Carter J, Evjen A, Zhang X, Shyr Y, Murphy BM, Cmelak AJ, Burkey BB, Netterville JL, Levy S, Yarbrough WG, Chung CH. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. Clin Cancer Res. 2006;12(3 Pt 1):701–709. doi:12/3/701 [pii]. 10.1158/1078-0432.CCR-05-2017.
141. Yarbrough WG, Whigham A, Brown B, Roach M, Slebos R. Phosphoinositide kinase-3 status associated with presence or absence of human papillomavirus in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys. 2007;69(2 Suppl):S98–101. doi:S0360-3016(07)00951-0 [pii]. 10.1016/j.ijrobp.2007.04.082.
142. Fury MG, Drobnjak M, Sima CS, Asher M, Shah J, Lee N, Carlson D, Wendel HG, Pfister DG. Tissue microarray evidence of association between p16 and phosphorylated eIF4E in tonsillar squamous cell carcinoma. Head Neck. 2011;33(9):1340–5. doi:10.1002/hed.21621.
143. Mamane Y, Petroulakis E, Martineau Y, Sato TA, Larsson O, Rajasekhar VK, Sonenberg N. Epigenetic activation of a subset of mRNAs by eIF4E explains its effects on cell proliferation. PLoS One. 2007;2(2):e242. doi:10.1371/journal.pone.0000242.
144. Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Mol Cell. 2003;12(4):889–901. doi:S1097276503003952 [pii].
145. Molinolo AA, Marsh C, El Dinali M, Gangane N, Jennison K, Hewitt S, Patel V, Seiwert TY, Gutkind JS. mTOR as a molecular target in HPV-associated oral and cervical squamous carcinomas. Clin Cancer Res. 2012;18(9):2558–68. doi:1078-0432.CCR-11-2824 [pii]. 10.1158/1078-0432.CCR-11-2824.
146. Yang SX, Nguyen D, Rubinstein L, Sherman ME, Swain SM, Tomaszewska JE, Doroshow JH. pAKT expression in paraffin-embedded xenograft tumors after fixation delays and human breast cancer by optimized immunohistochemistry. J Clin Oncol. 2012;30(Suppl):Abstr 10603.
147. Vezina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot (Tokyo). 1975;28(10):721–726.
148. Heitman J, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science. 1991;253(5022):905–9.
149. Rini BI. Temsirolimus, an inhibitor of mammalian target of rapamycin. Clin Cancer Res. 2008;14(5):1286–90. doi:14/5/1286 [pii]. 10.1158/1078-0432.CCR-07-4719.
150. Gabardi S, Baroletti SA. Everolimus: a proliferation signal inhibitor with clinical applications in organ transplantation, oncology, and cardiology. Pharmacotherapy. 2010;30(10):1044–56. doi:10.1592/phco.30.10.1044 [pii].
151. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008;30(2):214–26. doi:S1097-2765(08)00169-X [pii]. 10.1016/j.molcel.2008.03.003.
152. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res. 2007;67(22):10804–12. doi:67/22/10804 [pii]. 10.1158/0008-5472.CAN-07-2310.
153. Kalender A, Selvaraj A, Kim SY, Gulati P, Brule S, Viollet B, Kemp BE, Bardeesy N, Dennis P, Schlager JJ, Marette A, Kozma SC, Thomas G. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. Cell Metab. 2010;11(5):390–401. doi:S1550-4131(10)00084-7 [pii]. 10.1016/j.cmet.2010.03.014.
154. Bo S, Benso A, Durazzo M, Ghigo E. Does use of metformin protect against cancer in Type 2 diabetes mellitus? J Endocrinol Invest. 2012;35(2):231–5. doi:8327 [pii].
155. Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, Ueno Y, Hatch H, Majumder PK, Pan BS, Kotani H. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther. 2010;9(7):1956–67. doi:1535-7163.MCT-09-1012 [pii]. 10.1158/1535-7163.MCT-09-1012.
156. Ihle NT, Williams R, Chow S, Chew W, Berggren MI, Paine-Murrieta G, Minion DJ, Halter RJ, Wipf P, Abraham R, Kirkpatrick L, Powis G. Molecular pharmacology and antitumor

activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling. Mol Cancer Ther. 2004;3(7):763–72. doi:3/7/763 [pii].
157. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chene P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, Garcia-Echeverria C. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther. 2008;7(7):1851–63. doi:1535-7163.MCT-08-0017 [pii]. 10.1158/1535-7163.MCT-08-0017.
158. Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J, Baselga J. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res. 2008;68(19):8022–30. doi:68/19/8022 [pii]. 10.1158/0008-5472.CAN-08-1385.
159. Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, Jarrett C, Carter J, Murphy BA, Netterville J, Burkey BB, Sinard R, Cmelak A, Levy S, Yarbrough WG, Slebos RJ, Hirsch FR. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol. 2006;24(25):4170–6. doi:24/25/4170 [pii]. 10.1200/JCO.2006.07.2587.
160. Gupta AK, McKenna WG, Weber CN, Feldman MD, Goldsmith JD, Mick R, Machtay M, Rosenthal DI, Bakanauskas VJ, Cerniglia GJ, Bernhard EJ, Weber RS, Muschel RJ. Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. Clin Cancer Res. 2002;8(3):885–92.
161. Nathan CO, Liu L, Li BD, Abreo FW, Nandy I, De Benedetti A. Detection of the proto-oncogene eIF4E in surgical margins may predict recurrence in head and neck cancer. Oncogene. 1997;15(5):579–84. doi:10.1038/sj.onc.1201216.
162. Nathan CO, Amirghahari N, Abreo F, Rong X, Caldito G, Jones ML, Zhou H, Smith M, Kimberly D, Glass J. Overexpressed eIF4E is functionally active in surgical margins of head and neck cancer patients via activation of the Akt/mammalian target of rapamycin pathway. Clin Cancer Res. 2004;10(17):5820–7. doi:10.1158/1078-0432.CCR-03-0483. 10/17/5820 [pii].
163. Pattje WJ, Schuuring E, Mastik MF, Slagter-Menkema L, Schrijvers ML, Alessi S, van der Laan BF, Roodenburg JL, Langendijk JA, van der Wal JE. The phosphatase and tensin homologue deleted on chromosome 10 mediates radiosensitivity in head and neck cancer. Br J Cancer. 2010;102(12):1778–85. doi:10.1038/sj.bjc.6605707. 6605707 [pii].
164. Snietura M, Jaworska M, Mlynarczyk-Liszka J, Goraj-Zajac A, Piglowski W, Lange D, Wozniak G, Nowara E, Suwinski R. PTEN as a prognostic and predictive marker in postoperative radiotherapy for squamous cell cancer of the head and neck. PLoS One. 2012;7(3):e33396. doi:10.1371/journal.pone.0033396. PONE-D-11-18188 [pii].
165. Barbareschi M, Buttitta F, Felicioni L, Cotrupi S, Barassi F, Del Grammastro M, Ferro A, Dalla Palma P, Galligioni E, Marchetti A. Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas. Clin Cancer Res. 2007;13(20):6064–9. doi:13/20/6064 [pii]. 10.1158/1078-0432.CCR-07-0266.
166. Shinohara ET, Maity A, Jha N, Lustig RA. Sirolimus as a potential radiosensitizer in squamous cell cancer of the head and neck. Head Neck. 2009;31(3):406–11. doi:10.1002/hed.20898.
167. Janku F, Wheler JJ, Naing A, Falchook GS, Hong DS, Stepanek V, Fu S, Piha-Paul SA, Lee JJ, Luthra R, Tsimberidou AM, Kurzrock R. PIK3CA mutation H1047R is associated with response to PI3K/AKT/mTOR signaling pathway inhibitors in early phase clinical trials. Cancer Res. 2012. doi:0008-5472.CAN-12-1726 [pii]. 10.1158/0008-5472.CAN-12-1726.
168. Erlich RB, Kherrouche Z, Rickwood D, Endo-Munoz L, Cameron S, Dahler A, Hazar-Rethinam M, de Long LM, Wooley K, Guminski A, Saunders NA. Preclinical evaluation of dual PI3K-mTOR inhibitors and histone deacetylase inhibitors in head and neck squamous cell carcinoma. Br J Cancer. 2012;106(1):107–115. doi:10.1038/bjc.2011.495. bjc2011495 [pii].

169. Ramos AM, Fernandez C, Amran D, Sancho P, de Blas E, Aller P. Pharmacologic inhibitors of PI3K/Akt potentiate the apoptotic action of the antileukemic drug arsenic trioxide via glutathione depletion and increased peroxide accumulation in myeloid leukemia cells. Blood. 2005;105(10):4013–20. doi:2004-07-2802 [pii]. 10.1182/blood-2004-07-2802.
170. Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA. ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem. 2004;279(49):51323–30. doi:10.1074/jbc.M409565200. M409565200 [pii].
171. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med. 2002;8(1):35–40. doi:10.1038/nm0102-35. nm0102-35 [pii].
172. Kang Q, Cao Y, Zolkiewska A. Direct interaction between the cytoplasmic tail of ADAM 12 and the Src homology 3 domain of p85alpha activates phosphatidylinositol 3-kinase in C2C12 cells. J Biol Chem. 2001;276(27):24466–72. doi:10.1074/jbc.M101162200. M101162200 [pii].
173. Rao VH, Kandel A, Lynch D, Pena Z, Marwaha N, Deng C, Watson P, Hansen LA. A positive feedback loop between HER2 and ADAM12 in human head and neck cancer cells increases migration and invasion. Oncogene. 2012;31(23):2888–98. doi:10.1038/onc.2011.460. onc2011460 [pii].
174. Erjala K, Sundvall M, Junttila TT, Zhang N, Savisalo M, Mali P, Kulmala J, Pulkkinen J, Grenman R, Elenius K. Signaling via ErbB2 and ErbB3 associates with resistance and epidermal growth factor receptor (EGFR) amplification with sensitivity to EGFR inhibitor gefitinib in head and neck squamous cell carcinoma cells. Clin Cancer Res. 2006;12(13):4103–11. doi:12/13/4103 [pii]. 10.1158/1078-0432.CCR-05-2404.
175. Hatakeyama H, Cheng H, Wirth P, Counsell A, Marcrom SR, Wood CB, Pohlmann PR, Gilbert J, Murphy B, Yarbrough WG, Wheeler DL, Harari PM, Guo Y, Shyr Y, Slebos RJ, Chung CH. Regulation of heparin-binding EGF-like growth factor by miR-212 and acquired cetuximab-resistance in head and neck squamous cell carcinoma. PLoS One. 2010;5(9): e12702. doi:e12702 [pii]. 10.1371/journal.pone.0012702.
176. Knowles LM, Stabile LP, Egloff AM, Rothstein ME, Thomas SM, Gubish CT, Lerner EC, Seethala RR, Suzuki S, Quesnelle KM, Morgan S, Ferris RL, Grandis JR, Siegfried JM. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. Clin Cancer Res. 2009;15(11):3740–50. doi:1078-0432.CCR-08-3252 [pii]. 10.1158/1078-0432.CCR-08-3252.
177. Seiwert TY, Jagadeeswaran R, Faoro L, Janamanchi V, Nallasura V, El Dinali M, Yala S, Kanteti R, Cohen EE, Lingen MW, Martin L, Krishnaswamy S, Klein-Szanto A, Christensen JG, Vokes EE, Salgia R. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. Cancer Res. 2009;69(7):3021–31. doi:10.1158/0008-5472.CAN-08-2881 [pii].
178. Xu H, Stabile LP, Gubish CT, Gooding WE, Grandis JR, Siegfried JM. Dual blockade of EGFR and c-Met abrogates redundant signaling and proliferation in head and neck carcinoma cells. Clin Cancer Res. 2011:17(13):4425–38. doi:10.1158/1078-0432.CCR-10-3339. 1078-0432.CCR-10-3339 [pii].
179. Zhang YE. Non-Smad pathways in TGF-beta signaling. Cell Res. 2009;19(1):128–39. doi:cr-2008328 [pii]. 10.1038/cr.2008.328.
180. Bedi A, Chang X, Noonan K, Pham V, Bedi R, Fertig EJ, Considine M, Califano JA, Borrello I, Chung CH, Sidransky D, Ravi R. Inhibition of TGF-beta enhances the In vivo antitumor efficacy of EGF receptor-targeted therapy. Mol Cancer Ther. 2012. doi:1535-7163.MCT-12-0101-T [pii]. 10.1158/1535-7163.MCT-12-0101-T.
181. Clarke PA, Workman P. Phosphatidylinositide-3-kinase inhibitors: addressing questions of isoform selectivity and pharmacodynamic/predictive biomarkers in early clinical trials. J Clin Oncol. 2012;30(3):331–3. doi:10.1200/JCO.2011.38.7167 [pii].

Chapter 8
Jak/STAT Signaling in HNC

Bhavana S. Vangara and Jennifer R. Grandis

Abstract The Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway relays signals from cytokine receptors and receptor tyrosine kinases to the nucleus, thereby altering the expression of genes regulating normal cell functions, including growth, differentiation, and apoptosis. Constitutive Jak/STAT activation has been detected in most epithelial malignancies including head and neck cancer (HNC). STAT3 activation in HNC alters cell cycle progression, inhibits apoptosis, and facilitates proliferation and survival of cancer cells. Inhibition of aberrant STAT3 by a variety of strategies has been shown to abrogate HNC growth in vitro and in vivo suggesting that clinical approaches to block STAT3 activation may be beneficial in this cancer. Understanding the consequences of Jak/STAT pathway mutation is integral to developing and improving targeted therapies for HNC. In this chapter, we will review Jak and STAT inhibitors presently under development in preclinical models as well as treatments further along the pipeline that have entered clinical investigation.

Keywords Head and neck cancer · Squamous cell carcinoma · Jak · STAT · Targeted therapies

8.1 Overview of Jak/STAT

The Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway was first described in studies of interferon signaling where information from extracellular polypeptides, primarily growth factors, and cytokines, was relayed to cytoplasmic transcription factors that altered nuclear gene expression [12], [18], [35].

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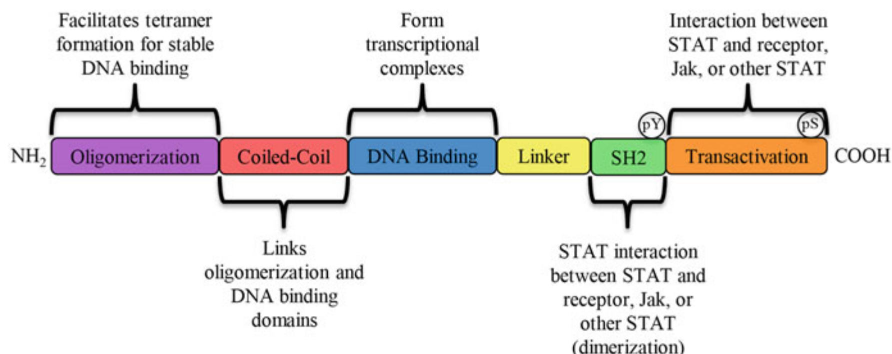

Fig. 8.1 Topology of the six major STAT functional domains. STAT functional domains are outlined including: *SH2* Src homology 2, NH_2 amino terminus, *COOH* carboxy terminus, *pY* phosphorylation site regulating DNA binding, and *pS* phosphorylation site, absent in STAT2 and STAT4

Jak/STAT signaling regulates several key physiological responses including cellular proliferation, development, differentiation, apoptosis, and inflammation [36], [46]. To date, four distinct mammalian Jak molecules (Jak1, Jak2, Jak3, Tyk2) and seven different STATs (STAT1–4, STAT5a, STAT5b, STAT6) have been identified.

8.2 Mechanism of Jak/STAT Pathway Activation

Jak proteins are 110–140 kDa nonreceptor tyrosine kinases constitutively bound to the intracellular domains of cytokine receptors. Jaks were named after Janus, a two-faced Roman god, because they possess two tyrosine kinase domains, one kinase (JH1) active at the C-terminus and another catalytically inactive pseudokinase domain (JH2) [11]. Five other Jak-homologous (JH) domains have been identified in members of the Jak family: JH3–JH5 (Src homology-2 (SH2)-line domain) and JH6–JH7 (FERM domain required for Jak-receptor interaction) [11].

X-ray crystallography supports shared domain homology among various STAT proteins. STATs are composed of an N-terminal domain responsible for STAT dimer interactions, a coiled-coil domain, a DNA-binding domain that forms transcriptional complexes, a linker domain, an SH2 domain where two activated STAT monomers dimerize at reciprocal phosphotyrosine residues, and a C-terminal transactivation domain [43] (Fig. 8.1).

Canonical Jak/STAT pathway signaling is initiated upon ligand binding to extracellular receptor domains, triggering receptor dimerization/oligomerization or rearrangement of preformed dimers [11] (Fig. 8.2). The conformational changes in the receptors bring the C-terminal JH1 kinase domains in close proximity and allow for rapid transphosphorylation of Jaks. Activated Jak proteins then phosphorylate tyrosine residues on receptor tyrosine tails. The phosphorylated tyrosines on Jaks and cytokine receptors recruit STAT proteins that bind the receptor and

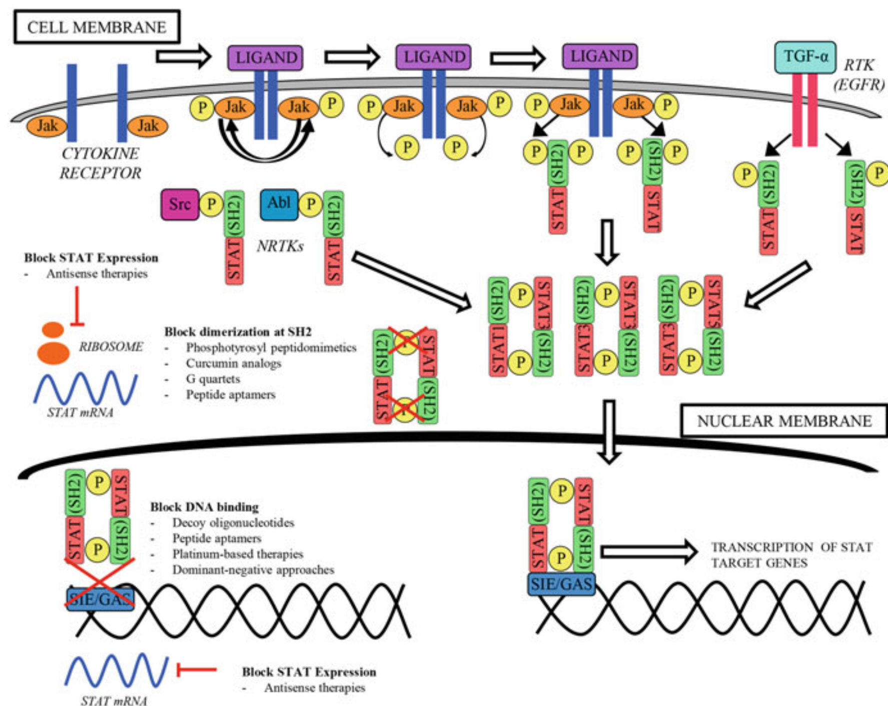

Fig. 8.2 Jak/STAT signaling and molecular inhibitors of STATs. Cytokine receptors with constitutively associated Jaks dimerize following ligand binding and subsequently transphosphorylate Jaks. STAT proteins are recruited to the phosphotyrosine residues of the receptor and bind via the SH2 domain. Activated STATs form homo- or heterodimers at their SH2 domains and translocate to the nucleus. The STAT dimers bind regulatory elements, including SIE/GAS, which leads to the transcription of STAT target genes. Binding of ligands to RTKs, such as EGFR, and NRTKs, such as Src and Abl, also activates STATs and initiates a downstream cascade. *Jak* Janus kinase, *STAT* signal transducer and activator of transcription, *SH2* Src homology 2, *SIE* serum-inducible element, *GAS* Gamma-interferon activating sequence, (*N*)*RTK* (non)receptor tyrosine kinase, *EGFR* epidermal growth factor receptor, *TGF-α* transforming growth factor-alpha, *Src* v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog, *Abl* V-Abl Abelson murine leukemia viral oncogene homolog

subsequently undergo phosphorylation at single tyrosine residues within the SH2 domain. Phosphorylated STATs dimerize and translocate to the nucleus to regulate expression of downstream target genes [12]. Noncanonical mechanisms involving unphosphorylated STAT1 and STAT3 signaling have been reported as well as STAT3-mediated NF-κB activation [68], [69].

Regulation of STAT proteins is a balance between positive and negative regulators of magnitude and duration of signaling. Activated STATs upregulate their expression in a positive-feedback mechanism following activation-induced STAT degradation [41]. Negative Jak/STAT pathway regulators include the SH2-containing phosphatase (SHP) which inactivates Jak, the protein inhibitor of activated STAT (PIAS), the suppressor of cytokine signaling (SOSC), and the protein tyrosine phosphatase (PTP) [35], [41].

8.3 Jak/STAT in Oncogenesis

Drosophila melanogaster models of gain-of-function Jak/STAT signaling first revealed the pathway's role in malignant neoplasia [20]. In 2005, a single point mutation in Jak2 JH2 pseudokinase domain (Val617Phe) was implicated in $> 80\,\%$ of a polycythemia vera patient cohort and was subsequently identified in other myeloproliferative disorders including essential thrombocythemia and myeloid metaplasia with myelofibrosis [26], [33], [40]. Although members of the Jak family have been associated with a number of cancers, direct evidence linking Jak mutations to HNC has not been reported.

Mutated and constitutively active forms of all seven STATs have been implicated in an array of human cancer cell lines and primary tumors, including hematological and solid malignancies such as HNC, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), pancreatic, colorectal, breast cancer, and cervical cancer ([9], [42]). Specifically, STATs 1, 3, 5a, and 5b have been indicated in head and neck tumorigenesis.

8.3.1 STATs in HNC

STAT1 is regarded as a tumor suppressor protein as it is involved in growth arrest and apoptosis whereas STAT3, STAT5a, and STAT5b are considered potential oncogenes due to their regulation of cyclin D1, cyclin D2, c-myc, Bcl-X_L, and survivin, genes encoding cell proliferation and survival [70]. STAT3 also modulates the expression of proteins responsible for angiogenesis, such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 (HIF-1), negatively regulates the expression of p53, and inhibits proinflammatory chemokines and cytokines responsible for antitumor responses [70].

8.3.2 STAT1 in HNC

IFN-γ-mediated STAT1 activation leads to apoptosis through Fas signaling and p21/WAF1-mediated cell cycle arrest [10]. STAT1's tumor suppressive function is best described in the context of human breast cancers where elevated phosphotyrosine STAT1 expression is associated with increased survival [64]. Conversely, STAT1 inhibition enhances HNC cell growth, and the loss of STAT1 heterozygosity may be predictive of HNC recurrence and death ([25], [67]).

8.3.3 *STAT3 in HNC*

8.3.3.1 In Vitro Preclinical Evidence of STAT3 in HNC

Constitutive activation of STAT3 homodimers (SIF-A) and STAT1/STAT3 heterodimers (SIF-B) has been identified in HNC cell lines and tumors. Elevated expression and phosphorylation levels of STAT3, but not STAT1, were detected in HNC cells compared to normal epithelial cells [18]. Dominant-negative mutant STAT3 constructs arrest growth, trigger apoptosis, and decrease downstream effects of STAT activation in HNC cell lines [18], [19], [29].

Several signal transduction pathways are involved in STAT3-mediated HNC. Activation of the α7 nicotinic receptor in oral keratinocytes initiates the Jak2/STAT3 pathway and the Ras/RAF1/MEK1/ERK cascade in tobacco-mediated HNC [4]. The epidermal growth factor receptor (EGFR) is a type I receptor tyrosine kinase known to recruit and activate SIF-A and SIF-B to induce Jak/STAT signaling following interaction with its ligand, TGF-α. Treatment of HNC cells with TGF-α leads to increased STAT3 phosphorylation and expression [18]. Studies in HNC cell lines demonstrate STAT3 levels correlate with EGFR mRNA and proteins levels, but not TGF-α mRNA levels, indicating that STAT3 activation is largely limited by EGFR expression [29].

8.3.3.2 In Vivo Preclinical Evidence of STAT3 in HNC

Increased STAT3 expression in primary HNC tumors and histologically normal mucosa from HNC patients suggests that STAT3 activation may serve as an early event in carcinogenesis [19]. Furthermore, a study concerning smokeless tobacco-mediated HNC demonstrated that 82 % of tumor samples from 90 patients reflected increased STAT3 expression with no detectable STAT3 in normal tissues [48]. STAT3 levels were highest in poorly differentiated HNC tumor samples, whereas STAT1 levels were highest in well-differentiated tumors. Thus, STAT1:STAT3 expression has been suggested as a prognostic indicator of HNC [3]. Immunohistochemistry staining for phosphorylated STAT3 in human squamous cell cancer of the tongue indicate that elevated expression of activated STAT3 correlate with nodal metastases, later clinical staging, and overall poor patient prognosis [45]. Additionally, decreased Bcl-X_L expression was observed in HNC xenograft models following STAT3 antisense treatment [19].

Elevated mRNA and protein expression levels of EGFR have been noted in $>$ 90 % of HNC tumors [17]. EGFR gene amplification has been reported in up to 30 % of HNC tumors and serves as an early biomarker for carcinogenesis. Increased EGFR expression has been correlated to decreased drug and radiotherapy response in HNC [14]. Additionally, c-Met and its ligand, the hepatocyte growth factors (HGF), are both overexpressed in HNC and initiate a similar pathway as EGFR causing enhanced motility, invasion, and angiogenesis [30]. In this manner, targeting c-Met in addition to EGFR may prevent acquired anti-EGFR resistance and enhance overall tumoricidal effects.

Notably, IL-6/gp130 interaction may activate STAT3. IL-6 serum levels were significantly elevated in patients with primary HNC compared to healthy controls and correlated with tumor staging [51]. STAT3 phosphorylation was abrogated following IL-6 immunodepletion in HNC cell lines [56]. Thus, IL-6 autocrine or paracrine activation of Jak1 and Jak2 leading to STAT3 phosphorylation may contribute to HNC tumorigenesis.

8.3.4 STAT5 in HNSCC

In addition to STAT3, STAT5 activation contributes to head and neck squamous cell carcinoma (HNSCC) oncogenesis by enhancing tumor growth, invasion, and epithelium-to-mesenchymal transition [32]. This study also demonstrated that STAT5 activation resulted in resistance to cisplatin and erlotinib therapy. Jak/STAT activation through the erythropoietin/Jak2/STAT5a cascade is also implicated in HNC invasion in vitro. Clinically, patients with solid tumors treated with erythropoietin-stimulating agents report decreased survival rates and increased recurrence rates [22], [35]. HNC xenograft models of enhanced STAT5b activation exhibit increased tumor growth [65].

8.4 Therapies Targeting STAT

STAT3 involvement in the progression of HNC, NSCLC, and other solid tumors have made targeting STAT3 a more prominent focus than STAT1 and STAT5. Four main strategies to inhibit STAT3 signaling are currently under development in several cancers including HNC: SH2, DNA binding, and N-terminal domain targets, and oligonucleotide-based approaches. Indirect STAT inhibition results from treatments in current clinical use, such as EGFR-directed monoclonal antibodies (mAb).

8.4.1 Preclinical Studies: Targeting the SH2 Domain

STAT dimerization and receptor interaction via phosphotyrosine motifs may be inhibited through SH2 domain blockade. Phosphotyrosyl peptidomimetics derived from a STAT3 SH2 domain sequence have been shown to disrupt SIF-A dimer formation, induce apoptosis, and inhibit cell growth in Src-transformed fibroblasts with persistent STAT3 activation ([58], [59]).

A curcumin analogue, FLLL32, functions by targeting the SH2 domain of STAT3 to limit phosphorylation and subsequent STAT3 nuclear activity. FLLL32 treatment enhances apoptosis in cisplatin-sensitive HNC cells and similar apoptotic rates were observed in FLLL32-sensitized cisplatin-resistant HNC cells treated with fourfold

less cisplatin compared to cisplatin monotherapy [1]. However, phase I studies revealed limited bioavailability of curcumin in human subjects [55].

A modified yeast two-hybrid screen was employed to identify short peptides (aptamers) that bind to the SH2 domain, the SH2-surrounding dimerization domain, or the DNA-binding domain of STAT3 [8], [47]. Peptide aptamers targeting STAT3 abrogated Bcl-X_L transcription and caused caspase-dependent apoptosis in murine melanoma and human myeloma cells [47].

Nonpeptide, small molecule SH2 inhibitors may represent a more practical approach to inhibiting STAT3 dimerization and a means to overcome shortcomings in cell permeability, in vivo stability, and potential immunogenicity. Several nonpeptide SH2 inhibitors designed using structure-based computational modeling are being evaluated. Recently, oral administration of one molecule, Stattic (STAT3 Inhibitory Compound), was shown to decrease HNC tumor growth and increase radiosensitivity by diminishing STAT3-mediated HIF-1α expression in a murine orthotopic model [2].

8.4.2 Preclinical Studies: Targeting the DNA-Binding and N-terminal Domains

CPA-27, a platinum (IV) compound, was found to inhibit STAT3–DNA binding at low micromolar concentrations in vitro; the significance of this interaction was corroborated in vivo when CPA-27 caused tumor regression in a CT26 colon cancer model [60]. Another compound, IS3 295, decreased the expression of cyclin D1 and Bcl-X_L, two known STAT3-regulated genes, and induced G_0/G_1 cell growth arrest and apoptosis in fibroblasts [61].

The N-domain present in all seven STATs is a 130-amino acid region comprising eight helices that mediates DNA and transcriptional machinery binding [6]. Synthetic cell-permeable derivatives of STAT3 N-domain helix 2 fused to protein transduction motifs selectively bind and inhibit the STAT3 N-domain as detected by FRET analysis [57]. While in vitro results appear promising, the inhibitors' method of action has yet to be elucidated.

8.4.3 Preclinical Studies: Oligonucleotide-Based Approaches Inhibiting STAT3 and STAT5

Recent advancements in nucleic acid-based strategies to target STAT proteins reveal promising preliminary results and render this method a plausible approach to block STAT activation. Antisense oligonucleotides, small interfering RNA (siRNA), dominant-negative constructs, and decoy oligonucleotide comprise current nucleic acid-based STAT inhibition techniques.

8.4.3.1 Antisense Approaches

Antisense RNA and siRNA effectively block aberrant STAT expression in preclinical cancer models [15], [23], [31], [50]. In the antisense RNA method, ~18–21-nucleotide-long RNA sequences are introduced into cells and bind complementary mRNA sequences in the cytoplasm or nucleus. Steric inhibition of ribosomal entry or cleavage of the oligonucleotide-mRNA complex by endogenous RNase H enzyme results in decreased gene expression [13]. Unfortunately, elevated liver enzymes and liver failure manifesting from toxicity to treatment are major setbacks to the clinical implementation of present antisense therapies.

siRNA knockdown is a formidable method of decreasing STAT3 expression in preclinical murine models of laryngeal, breast, pancreatic, and colorectal cancer [16], [23], [34], [50]. Additionally, STAT5 siRNA treatment in a hepatocellular carcinoma xenograft model resulted in increased growth inhibition and apoptosis [71]. However, the clinical application of the siRNA approach of STAT expression is limited to date due to barriers in drug delivery.

8.4.3.2 Dominant-Negative Approaches

STAT proteins are present as several isoforms. The α isoform represents the full-length protein. The β isoform, a truncated protein lacking the C-terminal transactivation domain of STAT3 and STAT5, has been experimentally manipulated to limit STAT activity through a dominant-negative approach [24], [43]. STAT3D, another dominant-negative form of STAT3, possesses E434A and E435A mutations, rendering it unable to bind DNA and its stable expression in HNC cell lines resulting in decreased growth rates [45], [49]. HNC cells transfected with a dominant-negative carboxy-truncated STAT5b (STAT5b***Δ***754) decreased cellular proliferation while Stat5a***Δ***750 failed to significantly alter HNC growth [37].

8.4.3.3 G-quartets Inhibit STAT3 Dimerization Through SH2 Domain

STAT3-specific guanine-rich deoxynucleotide sequences create G-quartet structures by forming two hydrogen bonds to the SH2 domain to destabilize and inhibit STAT3. A G-quartet oligonucleotide, T40214, impedes the growth of HNC tumor xenografts in mice over a 21-day period relative to placebo-treated mice [27], [28]. Furthermore, T40214 + paclitaxel-treated mice displayed a 35 % decrease in overall tumor size. In vitro analysis in three HNC cell lines: TU167, B4B8, and MDA1986 demonstrated that T40214 preferentially inhibits STAT3 DNA-binding activity over STAT1 activity. G-quartet oligonucleotides have demonstrated effectiveness in slowing tumor proliferation in animal models of NSCLC and prostate cancer [62], [63]. Current research in G-quartet oligonucleotides focuses on optimizing target selectivity and bioavailability in order to propel these forward as viable clinical treatments.

8.4.4 *Clinical Studies*

8.4.4.1 Indirect STAT Inhibition

In 2006, cetuximab, a chimeric immunoglobulin G1 (IgG1) EGFR mAb, became the first Food and Drug Administration (FDA) – approved molecular therapy to treat locoregional HNC in combination with radiotherapy and as a monotherapy for recurrent or metastatic HNC unresponsive to platinum-based chemotherapy [5]. Cetuximab binds at the endogenous ligand-binding site with high affinity to abrogate EGFR dimerization and autophosphorylation, preventing Jak/STAT pathway activation. Further, cetuximab is speculated to recruit natural killer cells and macrophages through antibody-dependent cell-mediated toxicity. Five-year survival in cetuximab/radiotherapy group was 45.6 % versus 36.4 % in the radiotherapy alone group [7]. Recently, constitutive activation of pSTAT3 has been shown to contribute to cetuximab resistance, and targeting STAT3 has been demonstrated as a viable method to abrogate EGFR resistance and augment cetuximab responses in HNC [53]. Additional EGFR inhibitors under investigation for HNC treatment include panitumumab, gefitinib, and erlotinib, as reviewed in detail in Chapter 4.

8.4.4.2 Direct STAT Inhibition

A transcription factor decoy approach was most recently employed to directly target STAT3 activity using a synthetic 15-mer double-stranded oligonucleotide that competitively binds *cis* elements within target genes to inhibit their expression [54]. The STAT3 decoy showed preferential STAT3 binding and arrested the proliferation of HNC cells in vitro [38]. Tumor growth inhibition and regression was seen in HNC xenograft models in mice following STAT3 intratumoral treatment [66]. The antiproliferative action of the STAT3 decoy has been demonstrated in other preclinical cancer models, including lung, breast, skin, and brain [53]. Furthermore, primates administered STAT3 decoy intramuscularly did not exhibit toxicity [52]. Although the STAT3 simultaneously abrogates STAT1 signaling, the STAT3 decoy independently inhibits HNC growth regardless of STAT1 levels or activation status [44].

The pharmacodynamic properties of the STAT3 decoy were tested in a phase 0 clinical trial in HNC patients. Patients received either a single intratumoral injection of 250, 500, or 1000 μg STAT3 decoy or saline control vehicle prior to tumor resection. Expression of STAT3 target genes cyclin D1 and Bcl-X_L significantly decreased when compared to pretreatment levels and to tumors receiving saline control. Additionally, no toxicities were noted and the maximum tolerated dose was not reached [54].

Systemic administration of the STAT3 decoy, however, failed to abrogate HNC xenograft tumor growth and did not decrease STAT3 target gene expression, demonstrating the requirement of local delivery of the decoy for antitumor effects. Degradation and thermal instability in vivo were suggested to explain the lack of

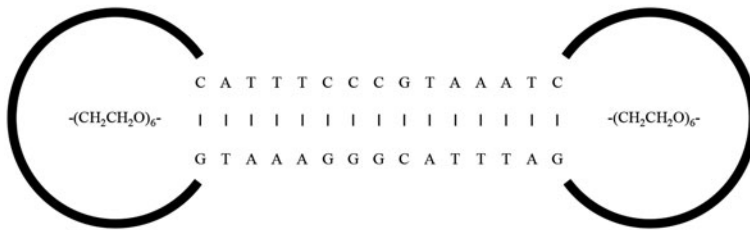

Fig. 8.3 STAT3 circular decoy oligonucleotide. The STAT3 decoy was modeled after the conserved hSIE genomic regulatory element present in the c-*fos* promoter. Eliminating free ends through circularization increased thermal stability of the STAT3 decoy and protected against degradation by nucleases. Systemic delivery of the optimized decoy oligonucleotide inhibited xenograft tumor growth and downstream STAT target gene expression

action of the STAT3 decoy when delivered intravenously. Thus, the parental STAT3 decoy was modified to adopt a cyclic structure, eliminating free ends susceptible to nucleases (Fig. 8.3). Systemic delivery of the newly formulated STAT3 decoy inhibited HNC xenograft tumor growth and STAT3 target gene expression [54]. The promising results from systemic administration of the cyclized STAT3 decoy underscore its potential as a therapeutic agent for STAT3-mediated HNC.

8.5 Therapies Targeting Jak

Although aberrant Jak activity is not a prominent method of oncogenesis in HNC, reports of frequent Jak mutations in other cancers have made Jak inhibition an active field of research. The identification of an acquired Jak2 mutation in hematological and myeloproliferative neoplasms has led to the rapid development of Jak2 inhibitors for use in preclinical and clinical studies [39]. Most Jak inhibitors in development are small-molecule, orally available kinase inhibitors categorized into class I (Jak2-specific) or class II (nonspecific) compounds [35].

Ruxolitinib (Jakafi®), an inhibitor of Jak1 and Jak2, was FDA approved in 2011 for the treatment of intermediate and high-risk myelofibrosis. Recently, the promising results of Jak2 inhibition in myeloproliferative disorders have made Jak inhibition an area of active investigation. A Jak2 inhibitor, AZD1480, was found to abrogate growth in human solid tumor cell lines and renal cell carcinoma xenografts with constitutive STAT3 activity [21]. A phase II trial investigating ruxolitinib in conjunction with capecitabine in the treatment of refractory metastatic pancreatic cancer is presently underway (ClinicalTrials.Gov.: NCT01423604).

8.6 Conclusions

Studies in HNC cell lines and human tumors identify the Jak/STAT pathway, specifically, STAT3 as a potential therapeutic target. While several Jak inhibitors (with secondary STAT3 inhibitory effects) are under clinical trial evaluation, few have been

examined in the context of HNC. Furthermore, apart from the STAT3 oligonucleotide decoy, STAT-targeted therapies have not advanced to clinical testing primarily due to lack of target selectivity and efficacy. Tumor heterogeneity is common in HNC and presents a substantial roadblock for the implementation of personalized therapy as multiple oncogenic pathways may be implicated in each cancer. Elucidating the role of the Jak/STAT pathway in HNC tumorigenesis will contribute to the development of more effective targeted approaches to treat HNC patients.

References

1. Abuzeid WM, Davis S, Tang AL, Saunders L, Brenner JC, Lin J, Fuchs JR, Light E, Bradford CR, Prince ME, Carey TE. Sensitization of head and neck cancer to cisplatin through the use of a novel curcumin analog. Arch Otolaryngol–Head Neck Surg. 2011;137(5):499–507. doi:10.1001/archoto.2011.63.
2. Adachi M, Cui C, Dodge CT, Bhayani MK, Lai SY. Targeting STAT3 inhibits growth and enhances radiosensitivity in head and neck squamous cell carcinoma. Oral Oncol. 2012. doi:10.1016/j.oraloncology.2012.06.006.
3. Arany I, Chen SH, Megyesi JK, Adler-Storthz K, Chen Z, Rajaraman S, Ember IA, Tyring SK, Brysk MM. Differentiation-dependent expression of signal transducers and activators of transcription (STATs) might modify responses to growth factors in the cancers of the head and neck. Cancer Lett. 2003;199(1):83–89.
4. Arredondo J, Chernyavsky AI, Jolkovsky DL, Pinkerton KE, Grando SA. Receptor-mediated tobacco toxicity: cooperation of the Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 pathways downstream of alpha7 nicotinic receptor in oral keratinocytes. FASEB J: Off Publ FedAm Soc Exp Biol. 2006;20(12):2093–2101. doi:10.1096/fj.06-6191com.
5. Astsaturov I, Cohen RB, Harari PM. EGFR-targeting monoclonal antibodies in head and neck cancer. Curr Cancer Drug Target. 2006;6(8):691–710.
6. Becker S, Groner B, Muller CW. Three-dimensional structure of the Stat3beta homodimer bound to DNA. Nature. 1998;394(6689):145–51. doi:10.1038/28101.
7. Bonner JA, Harari PM, Giralt J, Cohen RB, Jones CU, Sur RK, Raben D, Baselga J, Spencer SA, Zhu J, Youssoufian H, Rowinsky EK, Ang KK. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol. 2010;11(1):21–28. doi:10.1016/S1470-2045(09)70311-0.
8. Buerger C, Nagel-Wolfrum K, Kunz C, Wittig I, Butz K, Hoppe-Seyler F, Groner B. Sequence-specific peptide aptamers, interacting with the intracellular domain of the epidermal growth factor receptor, interfere with Stat3 activation and inhibit the growth of tumor cells. J Biol Chem. 2003;278(39):37610–21. doi:10.1074/jbc.M301629200.
9. Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A. STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol. 2003;197(2):157–168. doi:10.1002/jcp.10364.
10. Chin YE, Kitagawa M, Su WC, You ZH, Iwamoto Y, Fu XY. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science. 1996;272(5262):719–22.
11. Constantinescu SN, Girardot M, Pecquet C. Mining for JAK-STAT mutations in cancer. Trends Biochem Sci. 2008;33(3):122–131. doi:10.1016/j.tibs.2007.12.002.
12. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415–21.
13. Dean NM, Bennett CF. Antisense oligonucleotide-based therapeutics for cancer. Oncogene. 2003;22(56):9087–96. doi:10.1038/sj.onc.1207231.

14. Fung C, Grandis JR. Emerging drugs to treat squamous cell carcinomas of the head and neck. Expert Opin Emerg Drug. 2010;15(3):355–73. doi:10.1517/14728214.2010.497754.
15. Gao L, Zhang L, Hu J, Li F, Shao Y, Zhao D, Kalvakolanu DV, Kopecko DJ, Zhao X, Xu DQ. Down-regulation of signal transducer and activator of transcription 3 expression using vector-based small interfering RNAs suppresses growth of human prostate tumor in vivo. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2005;11(17):6333–41. doi:10.1158/1078-0432.CCR-05-0148.
16. Gao LF, Wen LJ, Yu H, Zhang L, Meng Y, Shao YT, Xu DQ, Zhao XJ. Knockdown of Stat3 expression using RNAi inhibits growth of laryngeal tumors in vivo. Acta Pharmacol Sin. 2006;27(3):347–52. doi:10.1111/j.1745-7254.2006.00277.x.
17. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res. 1993;53(15):3579–84.
18. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, Tweardy DJ. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro. J Clinical Investigation 1998;102(7):1385–92. doi:10.1172/JCI3785.
19. Grandis JR, Drenning SD, Zeng Q, Watkins SC, Melhem MF, Endo S, Johnson DE, Huang L, He Y, Kim JD. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci U. S Am. 2000;97(8):4227–32.
20. Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N. Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. EMBO J. 1995;14(12):2857–65.
21. Hedvat M, Huszar D, Herrmann A, Gozgit JM, Schroeder A, Sheehy A, Buettner R, Proia D, Kowolik CM, Xin H, Armstrong B, Bebernitz G, Weng S, Wang L, Ye M, McEachern K, Chen H, Morosini D, Bell K, Alimzhanov M, Ioannidis S, McCoon P, Cao ZA, Yu H, Jove R, Zinda M. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. Cancer Cell. 2009;16(6):487–97. doi:10.1016/j.ccr.2009.10.015.
22. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, Frommhold H. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. Lancet 2003;362 (9392):1255–60. doi:10.1016/S0140-6736(03)14567-9.
23. Huang C, Jiang T, Zhu L, Liu J, Cao J, Huang KJ, Qiu ZJ. STAT3-targeting RNA interference inhibits pancreatic cancer angiogenesis in vitro and in vivo. Int J Oncol. 2011;38(6):1637–44. doi:10.3892/ijo.2011.1000.
24. Huang Y, Qiu J, Dong S, Redell MS, Poli V, Mancini MA, Tweardy DJ. Stat3 isoforms, alpha and beta, demonstrate distinct intracellular dynamics with prolonged nuclear retention of Stat3beta mapping to its unique C-terminal end. J Biol Chem. 2007;282(48):34958–67. doi:10.1074/jbc.M704548200.
25. Ihle JN. The Stat family in cytokine signaling. Curr Opin Cell Biol. 2001;13(2):211–7.
26. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144–8. doi:10.1038/nature03546.
27. Jing N, Li Y, Xu X, Sha W, Li P, Feng L, Tweardy DJ. Targeting Stat3 with G-quartet oligodeoxynucleotides in human cancer cells. DNA cell Biol. 2003;22(11):685–96. doi:10.1089/104454903770946665.
28. Jing N, Zhu Q, Yuan P, Li Y, Mao L, Tweardy DJ. Targeting signal transducer and activator of transcription 3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer. Mol Cancer Ther. 2006;5(2):279–86. doi:10.1158/1535-7163.MCT-05-0302.
29. Kijima T, Niwa H, Steinman RA, Drenning SD, Gooding WE, Wentzel AL, Xi S, Grandis JR. STAT3 activation abrogates growth factor dependence and contributes to head and neck squamous cell carcinoma tumor growth in vivo. Cell growth & differentiation: the molecular biology journal of the American Association for. Cancer Res. 2002;13(8):355–62.

30. Knowles LM, Stabile LP, Egloff AM, Rothstein ME, Thomas SM, Gubish CT, Lerner EC, Seethala RR, Suzuki S, Quesnelle KM, Morgan S, Ferris RL, Grandis JR, Siegfried JM. HGF and c-Met participate in paracrine tumorigenic pathways in head and neck squamous cell cancer. Clin Ca Res. 2009;15(11):3740–50. doi:10.1158/1078-0432.CCR-08-3252.
31. Konnikova L, Kotecki M, Kruger MM, Cochran BH. Knockdown of STAT3 expression by RNAi induces apoptosis in astrocytoma cells. BMC cancer. 2003;3:23. doi:10.1186/1471-2407-3-23.
32. Koppikar P, Lui VW, Man D, Xi S, Chai RL, Nelson E, Tobey AB, Grandis JR. Constitutive activation of signal transducer and activator of transcription 5 contributes to tumor growth, epithelial-mesenchymal transition, and resistance to epidermal growth factor receptor targeting. Clin Ca Res. 2008;14(23):7682–90. doi:10.1158/1078-0432.CCR-08-1328.
33. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. A gain-of-function mutation of JAK2 in myeloproliferative disorders. New Engl J Med. 2005;352(17):1779–90. doi:10.1056/NEJMoa051113.
34. Kunigal S, Lakka SS, Sodadasu PK, Estes N, Rao JS. Stat3-siRNA induces Fas-mediated apoptosis in vitro and in vivo in breast cancer. Int J Oncol. 2009;34(5):1209–20
35. Lai SY, Johnson FM. Defining the role of the JAK-STAT pathway in head and neck and thoracic malignancies: implications for future therapeutic approaches. Drug Resist Updat. 2010;13(3):67–78. doi:10.1016/j.drup.2010.04.001.
36. Leeman RJ, Lui VW, Grandis JR. STAT3 as a therapeutic target in head and neck cancer. Expert Opin Biol Ther. 2006;6(3):231–41. doi:10.1517/14712598.6.3.231.
37. Leong PL, Xi S, Drenning SD, Dyer KF, Wentzel AL, Lerner EC, Smithgall TE, Grandis JR. Differential function of STAT5 isoforms in head and neck cancer growth control. Oncogene. 2002;21(18):2846–53. doi:10.1038/sj.onc.1205385.
38. Leong PL, Andrews GA, Johnson DE, Dyer KF, Xi S, Mai JC, Robbins PD, Gadiparthi S, Burke NA, Watkins SF, Grandis JR. (2003) Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci U S Am. 2003;100(7):4138–43. doi:10.1073/pnas.0534764100.
39. Levine RL, Gilliland DG. Myeloproliferative disorders. Blood. 2008;112(6):2190–8. doi:10.1182/blood-2008-03-077966.
40. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7(4):387–97. doi:10.1016/j.ccr.2005.03.023.
41. Li WX. Canonical and non-canonical JAK-STAT signaling. Trends Cell Biol. 2008;18(11): 545–1. doi:10.1016/j.tcb.2008.08.008.
42. Liang Z, Gao LH, Cao LJ, Feng DY, Cao Y, Luo QZ, Yu P, Li M. Detection of STAT2 in early stage of cervical premalignancy and in cervical cancer. Asian Pac J Trop Med. 2012;5(9): 738–42. doi:10.1016/S1995-7645(12)60117-5.
43. Lim CP, Cao X. Structure, function, and regulation of STAT proteins. Mol Biosyst. 2006;2(11):536–50. doi:10.1039/b606246f.
44. Lui VW, Boehm AL, Koppikar P, Leeman RJ, Johnson D, Ogagan M, Childs E, Freilino M, Grandis JR. Antiproliferative mechanisms of a transcription factor decoy targeting signal transducer and activator of transcription (STAT) 3: the role of STAT1. Mol Pharmacol. 2007;71(5):1435–43. doi:10.1124/mol.106.032284.
45. Masuda M, Suzui M, Yasumatu R, Nakashima T, Kuratomi Y, Azuma K, Tomita K, Komiyama S, Weinstein IB. Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. Cancer Res. 2002;62(12):3351–5.
46. Mertens C, Darnell JE Jr. SnapShot: JAK-STAT signaling. Cell. 2007;131(3):612. doi:10.1016/j.cell.2007.10.033.
47. Nagel-Wolfrum K, Buerger C, Wittig I, Butz K, Hoppe-Seyler F, Groner B. The interaction of specific peptide aptamers with the DNA binding domain and the dimerization domain of

the transcription factor Stat3 inhibits transactivation and induces apoptosis in tumor cells. Mol Cancer Res: MCR. 2004;2(3):170–82.
48. Nagpal JK, Mishra R, Das BR. Activation of Stat-3 as one of the early events in tobacco chewing-mediated oral carcinogenesis. Cancer. 2002;94(9):2393–400. doi:10.1002/cncr.10499.
49. Nakajima K, Yamanaka Y, Nakae K, Kojima H, Ichiba M, Kiuchi N, Kitaoka T, Fukada T, Hibi M, Hirano T. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J. 1996;15(14):3651–8.
50. Qian WF, Guan WX, Gao Y, Tan JF, Qiao ZM, Huang H, Xia CL. Inhibition of STAT3 by RNA interference suppresses angiogenesis in colorectal carcinoma. Braz J Med Biol Res. 2011;44(12):1222–30.
51. Riedel F, Zaiss I, Herzog D, Gotte K, Naim R, Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res. 2005;25(4):2761–5.
52. Sen M, Tosca PJ, Zwayer C, Ryan MJ, Johnson JD, Knostman KA, Giclas PC, Peggins JO, Tomaszewski JE, McMurray TP, Li C, Leibowitz MS, Ferris RL, Gooding WE, Thomas SM, Johnson DE, Grandis JR. Lack of toxicity of a STAT3 decoy oligonucleotide. Cancer Chemother Pharmacol. 2009;63(6):983–95. doi:10.1007/s00280-008-0823-6.
53. Sen M, Joyce S, Panahandeh M, Li C, Thomas SM, Maxwell J, Wang L, Gooding WE, Johnson DE, Grandis JR. Targeting Stat3 Abrogates EGFR Inhibitor Resistance in Cancer. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2012a;18(18):4986–96. doi:10.1158/1078-0432.CCR-12-0792.
54. Sen M, Thomas SM, Kim S, Yeh JI, Ferris RL, Johnson JT, Duvvuri U, Lee J, Sahu N, Joyce S, Freilino ML, Shi H, Li C, Ly D, Rapireddy S, Etter JP, Li PK, Wang L, Chiosea S, Seethala RR, Gooding WE, Chen X, Kaminski N, Pandit K, Johnson DE, Grandis JR. First-in-Human Trial of a STAT3 Decoy Oligonucleotide in Head and Neck Tumors: Implications for Cancer Therapy. Cancer Discov. 2012b. doi:10.1158/2159-8290.CD-12-0191.
55. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Ca Res. 2004;10(20):6847–54. doi:10.1158/1078-0432.CCR-04-0744.
56. Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. Cancer Res. 2003;63(11):2948–56.
57. Timofeeva OA, Gaponenko V, Lockett SJ, Tarasov SG, Jiang S, Michejda CJ, Perantoni AO, Tarasova NI. Rationally designed inhibitors identify STAT3 N-domain as a promising anticancer drug target. ACS Chem Biolo. 2007;2(12):799–809. doi:10.1021/cb700186x.
58. Turkson J, Ryan D, Kim JS, Zhang Y, Chen Z, Haura E, Laudano A, Sebti S, Hamilton AD, Jove R. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J Biol Chem. 2001;276(48):45443–55. doi:10.1074/jbc.M107527200.
59. Turkson J, Kim JS, Zhang S, Yuan J, Huang M, Glenn M, Haura E, Sebti S, Hamilton AD, Jove R. Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. Mol Cancer Ther. 2004a;3(3):261–9.
60. Turkson J, Zhang S, Palmer J, Kay H, Stanko J, Mora LB, Sebti S, Yu H, Jove R. Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent antitumor activity. Mol Cancer Ther. 2004b;3(12):1533–42.
61. Turkson J, Zhang S, Mora LB, Burns A, Sebti S, Jove R. A novel platinum compound inhibits constitutive Stat3 signaling and induces cell cycle arrest and apoptosis of malignant cells. J Biol Chem. 2005;280(38):32979–88. doi:10.1074/jbc.M502694200.
62. Weerasinghe P, Garcia GE, Zhu Q, Yuan P, Feng L, Mao L, Jing N. Inhibition of Stat3 activation and tumor growth suppression of non-small cell lung cancer by G-quartet oligonucleotides. Int J Oncol. 2007;31(1):129–36.
63. Weerasinghe P, Li Y, Guan Y, Zhang R, Tweardy DJ, Jing N. T40214/PEI complex: a potent therapeutics for prostate cancer that targets STAT3 signaling. Prostate. 2008;68(13):1430–42. doi:10.1002/pros.20807.

64. Widschwendter A, Tonko-Geymayer S, Welte T, Daxenbichler G, Marth C, Doppler W. Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2002;8(10):3065–74.
65. Xi S, Zhang Q, Gooding WE, Smithgall TE, Grandis JR. Constitutive activation of Stat5b contributes to carcinogenesis in vivo. Cancer Res. 2003;63(20):6763–71.
66. Xi S, Gooding WE, Grandis JR. In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: implications for cancer therapy. Oncogene. 2005;24(6):970–9. doi:10.1038/sj.onc.1208316.
67. Xi S, Dyer KF, Kimak M, Zhang Q, Gooding WE, Chaillet JR, Chai RL, Ferrell RE, Zamboni B, Hunt J, Grandis JR. Decreased STAT1 expression by promoter methylation in squamous cell carcinogenesis. J Natl Cancer Inst. 2006;98(3):181–9. doi:10.1093/jnci/djj020.
68. Yang J, Stark GR. Roles of unphosphorylated STATs in signaling. Cell Res. 2008;18(4):443–51. doi:10.1038/cr.2008.41.
69. Yang J, Chatterjee-Kishore M, Staugaitis SM, Nguyen H, Schlessinger K, Levy DE, Stark GR. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. Cancer Res. 2005;65(3):939–47.
70. Yu H, Jove R. The STATs of cancer–new molecular targets come of age. Nature reviews. Cancer. 2004;4(2):97–105. doi:10.1038/nrc1275.
71. Zhang L, Zhao Z, Feng Z, Yin N, Lu G, Shan B. RNA interference-mediated silencing of Stat5 induces apoptosis and growth suppression of hepatocellular carcinoma cells. Neoplasma. 2012;59(3):302–9. doi:10.4149/neo_2012_039.

Chapter 9
TGFβ Regulates EMT in Head and Neck Cancer

Jill M. Neiman and Xiao-Jing Wang

Abstract The epithelial-mesenchymal transition (EMT) has been a major focus of cancer research for many years, in part because of the association it has with metastasis and disease progression. In recent years, EMT has also been linked to increased cancer stem cell populations. The transforming growth factor-beta (TGFβ) pathway induces EMT in many cancer types, including head and neck cancer. There are several important transcription factors essential to TGFβ-induced EMT, including Snail and Twist. Additionally, several deregulated microRNAs contribute to the oncogenic process by influencing translation of these transcription factors. In this chapter, we will discuss the contribution of the TGFβ pathway, the transcription factors Snail and Twist, and the microRNAs involved in regulating EMT and cancer stem cell populations in head and neck cancer.

Keywords TGFβ · Snail · Twist · EMT · CSC · HNC · MicroRNA · Smad · Metastasis

9.1 Introduction

Epithelial-mesenchymal transition (EMT) is a complex process that is involved in metastasis and reoccurrence of many cancers, including head and neck cancer (HNC). Recent data have shown that EMT and increased cancer stem cell (CSC) populations are associated, and may help explain some of the characteristics of these particular tumor cells. Although EMT and CSCs have complex regulatory mechanisms, it has been shown that these processes in most cancers are mediated, in part, by the transforming growth factor-beta (TGFβ) signaling pathway. EMT induction by TGFβ is mediated through numerous factors, including Snail, Twist, and microRNAs (miRs).

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Additionally, many of these factors, as well as other key regulators, are involved in the EMT-associated link to increased CSC populations.

9.2 TGFβ Signaling Pathway

In many cancers, including HNC, the TGFβ pathway has been shown to contribute to tumorigenesis by causing increased EMT, angiogenesis, metastasis, and CSC populations. During carcinogenesis, TGFβ signaling has a dual role; in normal tissues and early tumorigenesis, TGFβ acts as a tumor suppressor that produces growth inhibition and promotes apoptosis [50]. However, as tumors become more advanced, mutations in the TGFβ pathway often lead to overexpression of the TGFβ ligand in addition to decreased tumor cell response to the ligand's suppressive effects. Overexpression of TGFβ also induces EMT in tumor cells, resulting in increased invasion and metastasis [102]. Furthermore, excessive TGFβ ligand in the tumor microenvironment causes other tumor promoting changes, including suppressed immune surveillance and increased angiogenesis.

TGFβ was first described in 1982 as an important mediator of the neoplastic transformation process, acting synergistically with TGFα or epithelial growth factor (EGF) to promote colony formation of normal rat kidney (NRK) cells in soft agar colony growth assays [5]. It was later shown that TGFβ pretreatment of mammary adenocarcinoma cells caused increased lung metastasis in a tail-vein injection rat model, likely through increasing the breakdown of the extracellular matrix (ECM) [111]. A few years later, TGFβ was found to induce EMT in normal and transformed mammary cells through a process requiring TGFβ receptor I kinase activity [65]. These studies highlight the importance of understanding the complex role of TGFβ signaling in tumor progression and metastasis.

In the canonical signaling pathway, TGFβ ligand binds to a heterotetrameric receptor complex, consisting of serine/threonine kinases, TGFβ receptors I and II (TGFβRI and TGFβRII, respectively). Ligand binding causes a conformational shift allowing the constitutively active TGFβRII to phosphorylate and activate TGFβRI [62], [66]. Activated TGFβRI phosphorylates the primary downstream signaling molecules in the TGFβ pathway, Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 can bind the common pathway mediator, Smad4, and translocate into the nucleus where they interact with other coactivators and corepressors to regulate gene transcription of growth inhibitors, apoptotic regulators, cellular adhesion molecules, and many other factors. One of the genes induced by TGFβ-ligand binding is the inhibitory Smad7, which creates a negative feedback loop on the TGFβ pathway (Fig. 9.1).

The Smad pathway is the predominant mechanism through which TGFβ acts to modify gene transcription; however, there are other noncanonical signaling pathways activated by TGFβ, including MAP kinase, Rho-GTPase, and PI3 kinase/AKT signaling pathways. Activation of these pathways can contribute to EMT and stem cell characteristics.

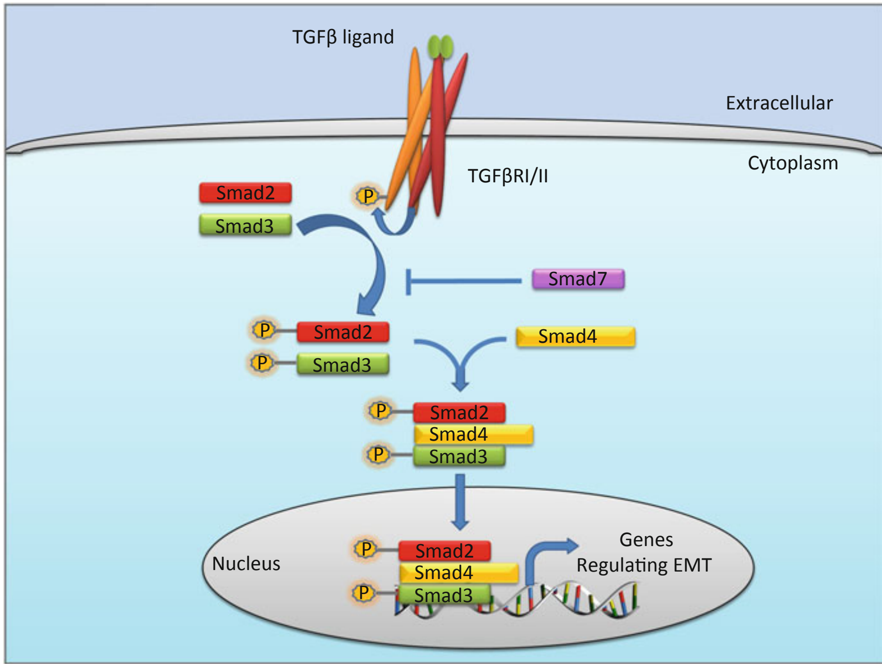

Fig. 9.1 The *TGFβ* pathway**.** Binding of TGFβ ligand to the *TGFβRI* and *TGFβRII* causes a conformational shift, allowing TGFβRII to phosphorylate TGFβRI. This receptor complex activates *Smad2/3* by phosphorylation, which is inhibited by *Smad7*. Once activated, Smad2/3 binds with the common *Smad4* and translocates into the nucleus. The Smad complex binds to promoters to regulate gene expression, including genes involved in *EMT* as well as the inhibitory Smad7

In HNC, alterations in TGFβ pathway regulation can occur through numerous mechanisms. Downregulation of TGFβRI and TGFβRII (53.8 and 28.8 %, respectively) is seen in head and neck squamous cell carcinoma (HNSCC) patient samples and correlates with increased invasion depth and lymph node metastasis [27], [110].

In addition to downregulation of the receptors, it is also common for tumors to have altered Smad signaling. Smad4 is frequently downregulated (86 %) as indicated by qRT-PCR of HNSCC patient samples [10]. Smad4 loss was found to be a tumor initiating and promoting event leading to genomic instability and inflammation. Additionally, Smad4 loss correlated with increased invasion, lymph node metastasis, and a worse clinical prognosis postoperatively and in TGFβ1-negative tumors ($p = 0.01$ and $p = 0.02$), although based on multivariate analysis, Smad4 expression alone cannot be used as an independent prognostic marker [28], [68]. Loss of Smad2 is seen in approximately 15–40 % of HNSCCs, and is correlated with increased local and distant metastasis, as well as disease reoccurrence [67], [114]. It was found that pSmad2/3 loss, seen in 8.5 % of patient samples, correlated with better outcomes than patients with active pSmad2/3 [113]. Unlike Smad2 and Smad4, Smad3 is typically preserved in HNSCC, and based on skin carcinogenesis studies in Smad3 germline knockout mice, may have a tumor promoting role related to altered TGFβ signaling in immune cells [51], [101].

Part of the mechanism involved in deregulation of the TGFβ pathway and loss of growth inhibition is overexpression of the TGFβ1 ligand, which is seen in 36–43 % of HNC patient samples [27], [68]. TGFβ1 ligand overexpression can feedback in an autocrine fashion to induce EMT in tumor cells, while also acting as a paracrine factor to activate fibroblasts in the surrounding stroma, contribute to angiogenesis, and cause inflammation [53], [98].

9.3 EMT in HNC

EMT is a normal physiological process critical for proper embryogenesis and development, as well as adult wound healing [11], [102]. It allows cells to alter their morphology and polarity, break down cellular adhesion complexes, and digest extracellular matrix, ultimately resulting in cell migration to other locations in the body where they participate in reconstruction and repair [84]. EMT has naturally been one of the major focuses of cancer research in the past 20 years, in part because of the clear association it has with disease progression and metastasis. One of the key identifying markers of EMT is decreased epithelial cadherin (E-cadherin) expression, which results in breakdown of adherens junctions, modification of Rho GTPase function, and release of β-catenin from the adherens junction complex. In addition to decreased E-cadherin expression, increased vimentin expression, loss of polarity complexes including Crumbs protein homolog-3 (CRB3) and Lethal giant larvae-2 (LGL2), changes in cytoskeletal elements, and the ability to produce matrix metalloproteases (MMPs) are associated with EMT. Moreover, EMT in cancer is influenced by many contributing cytokines, transcription factors, and cellular adhesion proteins.

In HNC, numerous key components of EMT have been identified (Fig. 9.2). Nijkamp et al. showed low expression of the epithelial adhesion molecule E-cadherin and high expression of the mesenchymal intermediate filament vimentin were significantly associated with increased distant metastatic potential in HNSCC patients as compared to the remaining patients in the study (100 vs. 44 %, respectively) [72]. Furthermore, Snail family member zinc-finger transcription factor, Slug (Snail2), has been shown to cause upregulation of N-cadherin, a neuronal adhesion molecule normally not expressed in epithelial cells, and decreased expression of desmosomal proteins, including desmoplakin, in HNC [44]. In addition to Slug, another E-cadherin transcriptional repression factor, zinc-finger E-box-binding homeobox 1 (ZEB1), is associated with decreased E-cadherin and EMT in HNC [35].

Growth factors and growth factor receptors play a critical role in EMT induction. Studies have found 80–90 % of HNSCCs had overexpression of epidermal growth factor receptor (EGFR), which induced EMT, increased MMP-9 production, and decreased E-cadherin expression [86]. These changes were associated with increased cell motility, invasion, and a poor prognosis as indicated by decreased disease-free survival and local-regional relapse, and EFGR expression was shown to be an independent prognostic marker for these diagnostic indicators [2], [126].

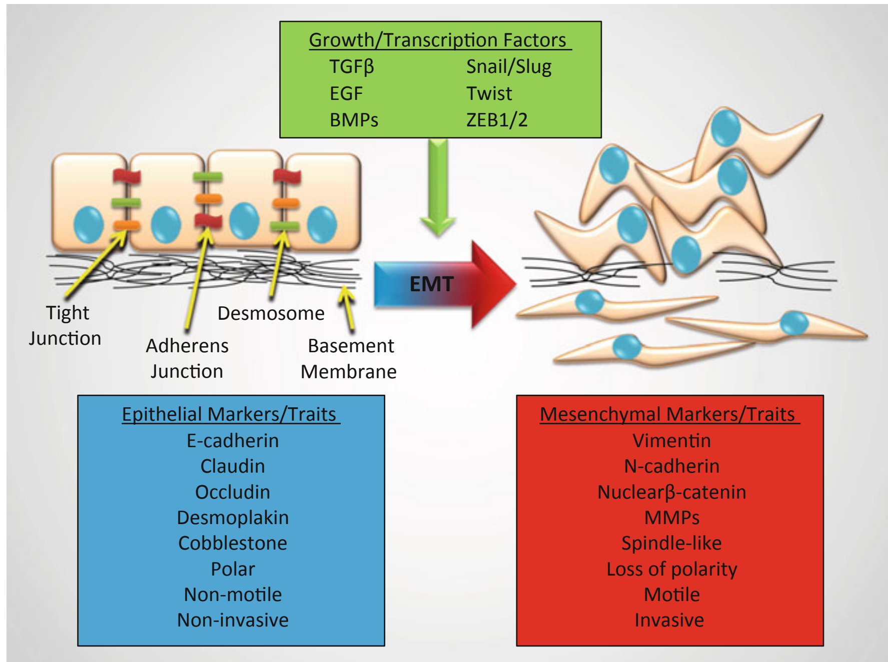

Fig. 9.2 Characteristics of *EMT*. Normal epithelial cells express epithelial markers and have numerous cell–cell connections including tight junctions, adherens junctions, and desmosomes. Additionally, these cells have cobblestone morphology and defined polarity. Overexpression of numerous growth and transcription factors contributes to EMT. After undergoing EMT, cells no longer express many of the epithelial factors, and instead express numerous mesenchymal markers. These cells also express proteases that breakdown the basement membrane, allowing them to invade. After EMT, cells have a spindle-like morphology and are more motile

Bone morphogenetic proteins (BMPs) have been associated with EMT and metastasis in HNSCC [32]. Immunohistochemistry (IHC) showed increased BMP-4 and p-Smad1, a downstream transcription factor in the BMP pathway, in samples from HNSCC patients with lymph node metastasis as compared to samples from patients without lymph node metastasis [83], [115]. In vitro studies show knockdown of Smad1 suppressed EMT induction by BMP4 and caused decreased invasion and migration. In addition to the above described pathways, both the canonical and non-canonical TGFβ signaling pathways are involved in EMT induction in HNC (see Sects. 9.4 and 9.5).

9.4 Regulation of EMT by TGFβ Signaling in HNC

Activation of the TGFβ pathway in HNC causes tumor cells to adopt a mesenchymal phenotype, which is represented by loss of E-cadherin, upregulation of mesenchymal markers, a spindle-like morphology, and increased motility. Activation of multiple cellular pathways is involved in this process.

TGFβ1 was initially shown to induce EMT in normal and transformed mammary epithelial cells, specifically through a TGFβRI-mediated mechanism [65]. It was soon found that TGFβ1 can induce EMT in virtually all epithelial cell types. More recently, Han et al. found that deletion of TGFβRII decreased TGFβ1 induced EMT [37], suggesting TGFβ1 induction of EMT through a receptor-dependent mechanism. In HNSCC, TGFβ1 overexpression has been shown to contribute to increased MMP-9 and breakdown of the extracellular matrix through regulation of myosin-light chain kinase, leading to increased ECM breakdown and invasion [56], [91].

Many TGFβ1-ligand-induced genes are regulated through the Smad pathway, which is a key component of TGFβ-mediated EMT. Petersen et al. showed knock-down of Smad3 caused increased latency and delayed bone metastasis in a breast cancer model. However, in this same model, Smad2 knockdown caused more aggressive metastasis than seen in the control, suggesting that Smad2 and Smad3 have divergent roles [80]. Additionally, Hoot et al. found that Smad2 ablation in mouse keratinocytes increased EMT and caused more poorly differentiated histology in skin SCC [39]. In contrast, the spontaneous HNSCC tumors that arise from knock-out of the common Smad4 resulted in tumors with increased genomic instability and inflammation; however, the tumors were well differentiated and did not show evidence of EMT as seen in Smad2 knockout tumors [10]. Further analyses revealed that Smad2 loss-associated EMT required increased Snail expression mediated by Smad3 and Smad4 [39]. These data suggest that Smads have distinct roles in EMT and tumor progression.

Smads can interact with other transcription factors to regulate gene expression and EMT. One important example of this is Smad interaction with ZEB proteins, including ZEB1 and ZEB2/SIP1. The ZEB transcription factors have a Smad-binding domain that can directly interact with Smads [20], [108]. However, Shirakihara et al. showed that E-cadherin repression by ZEB2 is independent of Smad binding. Furthermore, they showed that ZEB1 and ZEB2 were necessary for TGFβ-mediated EMT, and that induction of these factors was likely regulated through transcriptional activation by Smads [90].

Janda et al. showed the TGFβ- and Ras-signaling pathways work synergistically to induce EMT. They used Ras-transformed or nontransformed mammary epithelial cells in combination with Ras-pathway inhibitors to show that the Raf/MAPK activation was required for sustained TGFβ1-mediated EMT, tumorigenesis, and metastasis [42]. This pathway is important in HNCs as well. H-ras mutation occurs in 4–5 % of human HNCs [1], [97]. Further, Lu et al. found that 63 % of HNSCC patient samples had Ras overexpression with concurrent TGFβRII expression loss [57].

9.5 Noncanonical TGFβ Signaling in EMT

While the Smad pathway influences TGFβ-induced EMT, there are several other important mechanisms driving this tumor-promoting change independent of Smad activation. TGFβRII can phosphorylate the polarity protein PAR6, which causes

local degradation of the RhoA small GTPase and disassembly of intercellular tight junctions in mammalian cells [40], [76]. Additionally, TGFβRI binds to the ubiquitin ligase TRAF6, which mediates activation of several MAP kinases including TAK1, MAPKK3/6, and the Jun/p38 pathway [94], [116], [123]. However, Yu et al. showed that mammary tumor cells need active Smad signaling in addition to p38 pathway activation to achieve complete EMT [123]. In fact, elevated p38 levels have been seen in patient HNSCC samples [31]. This suggests that while noncanonical mechanisms of the TGFβ pathway contribute to EMT, Smad signaling likely works synergistically with these additional pathways to maintain a complete EMT phenotype.

9.6 Snail-mediated EMT in HNC

Snail is a master regulator of EMT, and is a transcriptional repressor of E-cadherin. Snail (Snail1) belongs to a family of transcription factors that have four to six zinc-finger domains that bind to E-box (5′-CACCTG-3′) regions located in the promoter region of target genes [71]. There are two other proteins in this family, Slug (Snail2) and Smuc (Snail3); however, Snail has the highest affinity for binding to E-box DNA domains and is a more potent inhibitor of E-cadherin gene transcription [9]. Snail represses the transcription of other epithelial genes including PTEN, Muc1, and the tight junction integral proteins claudin and occludin [41], [74], [79]. Additionally, Snail induction results in upregulation of mesenchymal markers, such as vimentin, through release of transcriptional factors that are retained by E-cadherin as well as interacting with β-catenin to promote transcriptional activation of Wnt target genes [92], [96].

Snail induction can occur through numerous mechanisms, one of which is activation of the TGFβ pathway. Activation of Snail by the TGFβ pathway requires Smad interaction with high mobility group A2 (HMGA2) protein, which is thought to enhance Smad binding to the Snail promoter [103]. Once induced, Snail, Smad3, and Smad4 can work cooperatively to form a transcriptional repressor complex that mediates EMT [109] (Fig. 9.3).

Mendelsohn et al. found that over 52 % of HNSCC patients' IHC samples stained positively for Snail, and this staining was inversely proportional to E-cadherin expression [64]. Additionally, it was found that positive Snail staining was associated with poor differentiation, a basaloid classification, lymphovascular invasion, and lymph node metastasis [64]. In vitro studies found increased Snail expression in oral SCC tumor lines, which was associated with decreased E-cadherin, and a more aggressive, metastatic phenotype [122]. Similarly, Usami et al. found esophageal SCC cell lines that overexpressed Snail also expressed the mesenchymal marker vimentin, but not E-cadherin or claudins. Furthermore, IHC of patient esophageal SCC samples showed that increased Snail staining at the invasive front corresponded with lymphatic and venous vessel invasion, lymph node metastasis, and advanced tumor stage [106]. Based on these studies, snail expression in HNC correlates with an EMT phenotype, increased metastasis, and may be a good prognostic marker for staging.

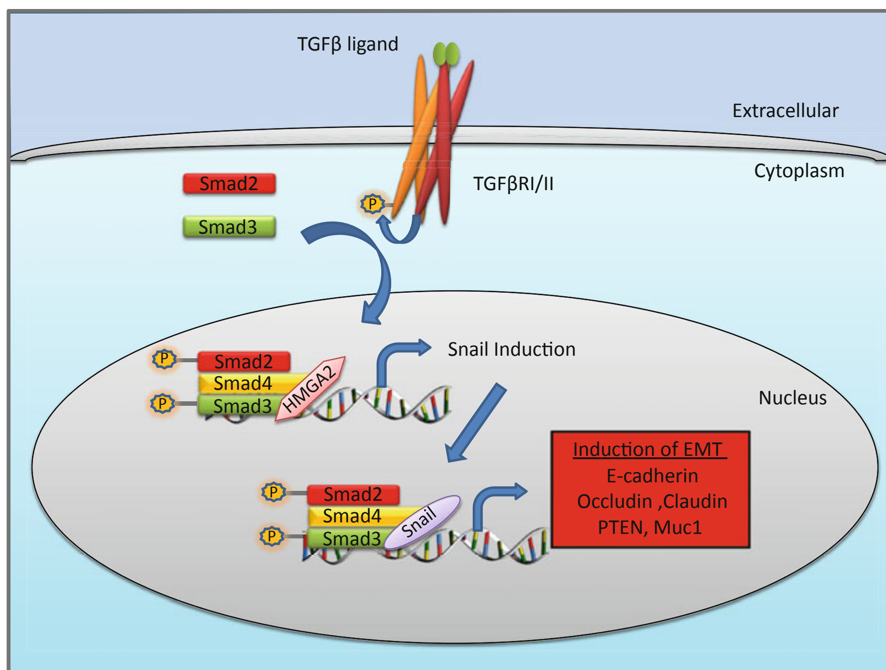

Fig. 9.3 The Snail pathway in *TGFβ*-mediated *EMT*. Binding of TGFβ ligand to the *TGFβRI* and *TGFβRII* causes activation and nuclear translocation of *Smads*. *HMGA2* forms a complex with Smads on the promoter of *Snail,* resulting in induction of Snail. Snail can form a repressor complex with *Smad3/4* that can downregulate numerous genes, including those involved in maintaining an epithelial phenotype. This contributes to TGFβ-induced EMT

In addition to modulating the EMT phenotype, Snail can alter the apoptotic response, possibly through downregulation of PTEN, p53, or BID [25], [43], [107]. Cell lines overexpressing snail showed decreased apoptosis in response to radiation and genotoxic drugs [25], [43]. Similarly, Dennis et al. found that induction of Snail was sufficient to cause a mesenchymal phenotype in HNSCC cell lines, and Snail overexpression conferred resistance to erlotinib, an EGFR tyrosine kinase inhibitor [24].

9.7 Twist Pathways in EMT of HNC

Twist proteins are part of a large family of basic helix-loop-helix transcription factors that were originally identified for binding to E-box responsive elements to regulate gene transcription [48], [121]. There are two Twist genes in vertebrates, Twist1 and Twist2, both of which are able to form homo- and heterodimers that bind the E-box regulatory domain [14]. Once bound, Twist proteins can act as transcriptional

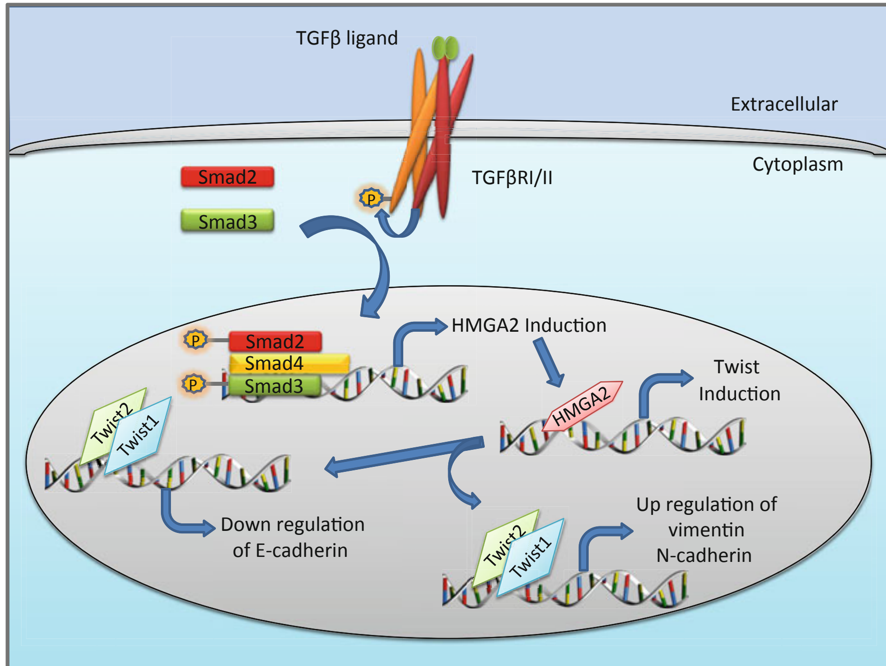

Fig. 9.4 Twist in *TGFβ*-mediated EMT. TGFβ receptor activation results in nuclear translocation of activated *Smads* where they induce *HMGA2* transcription. HMGA2 binds to the promoter region of *Twist,* resulting in upregulation. Twist can act as a repressor of epithelial markers and an inducer of mesenchymal markers

activators, but more commonly act as transcriptional repressors by either recruiting histone deacetylases, or inhibiting acetyl-transferases [36], [77]. Furthermore, Twist proteins can modify gene transcription by inhibiting the function of other transcription factors including NF-κB, p53, MyoD, and myocyte enhancer factor-2 (MEF2) [7], [88], [89], [95]. Having the ability to interact with other pathways and induce longer-acting transcriptional changes gives Twist proteins a diverse and multifaceted response to a given stimuli.

TGFβ signaling and Snail contribute to Twist regulation. Twist is a target of Snail repression, causing a transient decrease in Twist levels after initial TGFβ treatment [105]. However, as Snail levels normalize, Twist is induced, in part, through TGFβ induction of HMGA2 [104]. Twist1 is necessary for maintenance of TGFβ-induced EMT. Induction of Twist proteins contributes to EMT through two possible mechanisms. Twist proteins can form heterodimers that bind to the E-box domain in the promoter of E-cadherin, resulting in downregulation of E-cadherin [26]. Alternatively, Twist proteins may be more involved with upregulation of mesenchymal proteins, including vimentin and N-cadherin [55] (Fig. 9.4). In addition to their role in EMT regulation, Twist proteins can influence tumor initiation and progression through numerous other mechanisms. Some of these include overriding

oncogene-induced apoptosis or senescence, increasing chemotherapeutic resistance, and facilitating cancer cell invasion and metastasis [3], [15], [16], [52], [59], [117].

Overexpression of Twist proteins is commonly seen in cancers. In HNC, overexpression of Twist correlated with a high pathological grade, lymph node metastasis, disease progression, and a poor prognosis ($p < 0.001$) [30], [75], [119]. Additionally, Twist expression correlated with disease reoccurrence in oral SCC [112]. Based on these studies, Twist expression should be investigated for future use as a prognostic marker in HNC.

9.8 Regulation of CSCs Through EMT in HNC

In more recent years, researchers have discovered that tumors consist of a very heterogeneous cell population; a small subpopulation of these cells, termed CSCs or tumor-initiating cells, have two key properties—the ability to self-renew, and the ability to regenerate the heterogeneity of the parental tumor [19]. CSCs have been implicated in tumor progression, metastasis, drug resistance, and reoccurrence.

There are two predominant hypotheses on the origin of CSCs. CSCs can arise from the transformation of normal adult tissue stem cells that have accumulated numerous mutations, or they can develop from progenitor cells that have acquired mutations endowing the tumor cell with self-renewing capabilities [63]. Recently, Mani et al. identified EMT as another key regulator of CSC populations [60]. They found that induction of EMT by TGFβ treatment in mammary epithelial cells increased expression of CSC markers, and that transformed mammary epithelial cells that had undergone EMT were more able to form colonies in soft agar and tumors in mice as compared to transformed cells that did not undergo EMT induction. Additionally, they saw that CSCs isolated from mammary carcinomas expressed EMT markers, and EMT transcription factors including Snail and Twist were upregulated [60]. In SCC, Biddle et al. showed there are two distinct populations of CSC, one that is migratory with an EMT phenotype, and one that is proliferative with an epithelial phenotype [8].

Prince et al. were the first to show a CSC population in HNC-CD44 + cells. However, large numbers of these cells were necessary to initiate a tumor (> 5,000 cells) [82]. Another study showed that CD44- cells in HNC also had CSC properties, implying there may be numerous CSC populations within a given tumor [58], [73]. Research in recent years has focused on markers to identify these populations. Several other putative HNC stem cell markers identified include aldehyde dehydrogenase 1 (ALDH1), CD34, CD117, CD133, and Hoechst side population (SP) [45], [58], [81], [93], [124].

Yang et al. showed that in HNC, Twist1 overexpression resulted in upregulation of Bmi1, which caused induction of EMT, increased tumor-initiating capabilities, and increased expression of CSC markers. Furthermore, high levels of both Twist1 and Bmi1 were associated with decreased survival in HNC patients [118]. Another study found that increased ZEB1/2 coexpression was associated with poor survival

rates and an increase in the CD133 + CSC population [18]. These studies suggest that not only are EMT and CSC properties associated with each other, but they also can be used as prognostic indicators in HNC.

9.9 MicroRNA Contributions to EMT and CSCs in HNC

The role of miRs in cancer has received considerable attention in recent years. MiRs are small (18–24 nucleotide), noncoding RNAs that regulate posttranscriptional gene expression. They are transcribed as precursors that need to be processed by the DROSHA complex and exported to the cytoplasm by Exportin5 before they can interact with target mRNAs [6], [38]. MiRs regulate gene expression by binding to the 3′ UTRs of target mRNAs, causing either degradation of the mRNA by the RISC if it is an exact match, or translational repression in the case of an imperfect match [47]. In the past several years, researchers have begun to uncover the diverse role that miRs play in almost every biological process, including cancer, where they can have either oncogenic or tumor suppressive properties [29], [69].

Some miRs have been implicated in EMT (Fig. 9.5). Park et al. found that in ovarian cancer, the miR-200 family targets ZEB1 and ZEB2; hence, decreased expression of miR-200 resulted in ZEB-mediated E-cadherin repression, vimentin upregulation, and EMT induction [78]. In HNSCC, Lo et al. found decreased expression of miR-200c in regional lymph node metastasis, as well as increased Bmi1 expression. They showed Bmi1 was a target of miR-200c, and that overexpression of miR-200c or knockdown of Bmi1 inhibited CSC properties and caused decreased lung metastasis. Furthermore, they showed that overexpression of miR-200c decreased Snail, ZEB1, and N-cadherin expression [54]. Interestingly, it has been shown that in lung cancer, expression of the miR-200 family can be repressed by ZEB1 and ZEB2, suggesting a complex negative feedback loop [12]. TGFβ treatment induced EMT that was accompanied by decrease in the miR-200 family, elevated ZEB1/2 mRNA, and decreased E-cadherin. In fact, Gregory et al. found prolonged treatment with TGFβ caused DNA methylation of the miR-200 loci that correlated with decreased miR-200 levels [33], [34]. TGFβ regulation of both ZEB1 and altered miR processing plays a role in this double-feedback loop [22].

Reduced expression of miR Let-7 in combination with miR-205 has been associated with a poor prognosis in HNSCC ($p = 0.01$) [17]. Let-7 targets HMGA2, which is involved in regulating TGFβ induced Twist and Snail expression [49], [104]. Therefore, decreased Let-7 would alleviate inhibition on these two major regulators of EMT. Additionally, in pancreatic cancer, TGFβ1 treatment can repress mature Let-7 but not the precursor of Let-7 through MMP-14 [21], implying that the TGFβ pathway can impact miR expression at multiple levels.

Several studies have shown that miR-155 is increased in HNC. One possible mechanism of how this overexpression contributes to oncogenesis is through targeting the tumor suppressor and epithelial marker APC [85]. In breast cancer, miR-155 was shown to contribute to TGFβ-mediated EMT through targeting RhoA [46]. Further investigation of miRs and their contributions to EMT in HNC is warranted.

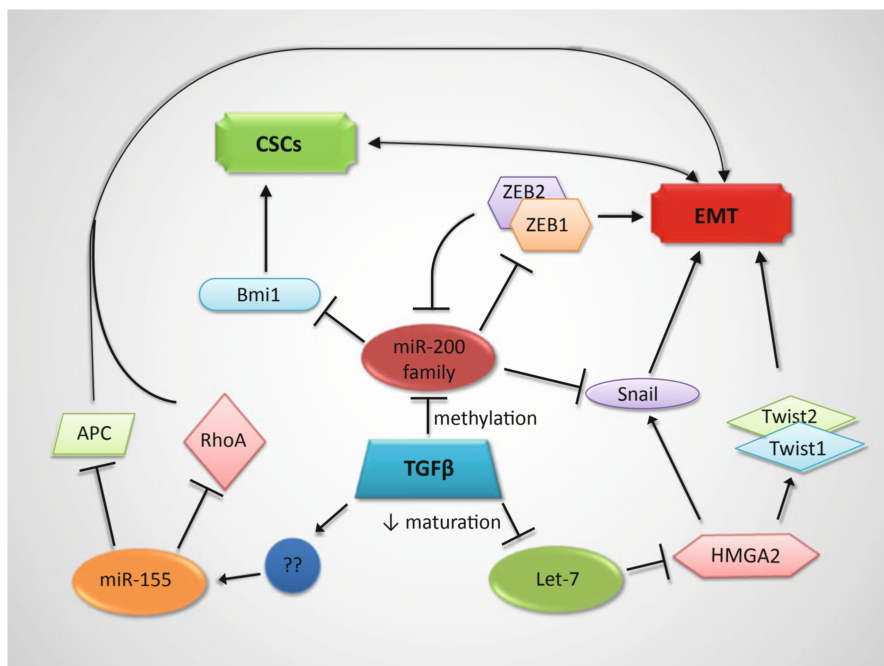

Fig. 9.5 *TGFβ* regulation of miRs involved in *EMT*. TGFβ causes methylation and inhibition of the *miR-200 family*. The miR-200 family targets several transcription factors that are involved in regulation of EMT and *CSCs*. TGFβ also decreases mature *Let-7*. Let-7 targets transcription factors involved in EMT. *MiR-155* is upregulated with TGFβ activation, although the mechanism is not clear. MiR-155 targets additional EMT regulators

9.10 Link Between EMT, CSCs, and Metastasis

EMT is often discussed as being linked to metastasis. In fact, there are numerous studies in HNC supporting this conclusion [70], [72], [87], [120], [126]. However, some recent data suggest that EMT and metastasis are not always associated. Han et al. found tumors containing deleted TGFβRII and TGFβ1 overexpression were still able to metastasize, despite maintaining E-cadherin expression, suggesting that alternative noncanonical pathways, such as the RhoA/Rac and MAPK, may play important roles [37]. Likewise, Lu et al. found that mouse models with K-ras activation and decreased TGFβRII expression developed HNSCC with 100 % penetrance, 35 % of which had lymph node metastasis, yet these tumors had not gone through EMT [57]. Similarly, Smad2 deficient tumors had increased EMT with decreased metastasis compared to Smad4 deficient tumors that had lung metastasis despite relatively decreased EMT [4], [39]. This suggests it is possible EMT and metastasis are not directly linked, and that the TGFβ pathway may be a key regulator of both these processes.

CSCs may be a better indicator of metastatic potential, although, as described previously, there is a clear link between CSCs and EMT. In HNC, CSCs isolated as CD44 + , ALDH1 + , and SP + all were shown to have increased metastasis as compared to non-CSC enriched tumor grafts [15], [23], [93], [99], [124]. Similarly, Biddle et al. showed that between two distinct CSC populations having either epithelial or mesenchymal phenotypes, only ALDH1 + cells were able to transit from a mesenchymal to epithelial morphology, implying that only ALDH + cells are able to seed a metastatic site [8]. While there appears to be an association between CSCs and metastasis, evidence for a clear mechanism linking these two remains elusive. Additional studies clarifying the contributions of EMT and CSCs to metastasis and tumor progression in HNC are necessary to provide additional insight into novel treatment approaches.

9.11 Implications for Therapy

Several of the molecules discussed in this chapter show promise as targets for treating HNSCC. Inhibiting the TGFβ pathway has recently become an exciting and promising therapeutic target in HNSCC. There are numerous inhibitors currently under investigation as anticancer therapeutics that are in discovery through Phase III trials. These drugs have one of four general approaches to impeding the TGFβ pathway including inhibition of TGFβ1 ligand synthesis, disruption of ligand-receptor interaction, inhibition of receptor activation, and inhibition of SMAD phosphorylation [13]. Of these therapies, some of the most advanced studies are in monoclonal antibodies, antisense oligonucleotides, and small ligand inhibitors, and to date, have shown few side effects with promising growth inhibition and decrease in metastasis.

While there are not many studies in HNSCC, one recent study showed that treatment with one of these inhibitors, GC-1008, caused decreased motility and invasion mediated by integrin downregulation in HNSCC cell lines [100]. This study suggests that TGFβ pathway inhibition may be a good molecular target in HNSCC as well as other cancers. There is some evidence that combination therapy of TGFβ inhibition with other pathway inhibitors, such as of the EGF pathway, may provide enhanced benefit [125].

Snail inhibition shows some promise as a molecular target as well, especially in combination treatment. Recent work has shown that HNSCC resistance to the commonly used EFGR inhibitor, gefitinib, shows acquisition of an EMT phenotype that is partially regulated by a compensatory increase in the AKT/GSK-3β/Snail pathway [61]. While there are not any Snail inhibitors currently in clinical trials, this study indicates that combination treatment of a Snail inhibitor with EGFR treatment may be an effective therapy against HNSCC and warrants further investigation.

To date, there are little data on anticancer therapies against Twist, ZEB, or the miRs discussed in this chapter; however, as these are key regulators of EMT, they show promise as potential targets and need additional studies to clarify if inhibiting these pathways may have therapeutic benefit. As EMT has also been shown to correlate with

CSC properties, it is important for future studies to examine the impact that inhibitors which have been designed to decrease EMT may have on regulating CSCs. These drugs may confer a particular benefit when used in combination with other therapies by decreasing the intrinsically resistant CSC population in addition to inhibiting EMT. As the field moves forward, modulation of EMT and CSC pathways through inhibiting TGFβ, Snail, Twist, or EMT-mediating miRs are promising candidates for successful combination treatment of HNSCC.

References

1. Agrawal, N, Frederick MJ, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–1157.
2. Ang KK, Berkey BA, et al. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res. 2002;62(24):7350–6.
3. Ansieau S, Bastid J, et al. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell. 2008;14(1):79–89.
4. Ansurudeen I, Willenberg HS, et al. Endothelial factors mediate aldosterone release via PKA-independent pathways. Mol Cell Endocrinol. 2009;300(1–2):66–70.
5. Anzano MA, Roberts AB, et al. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 1982;42(11):4776–8.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2): 281–97.
7. Bialek P, Kern B, et al. A twist code determines the onset of osteoblast differentiation. Dev Cell. 2004;6(3):423–35.
8. Biddle A, Liang X, et al. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res. 2011;71(15):5317–26.
9. Bolos V, Peinado H, et al. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci. 2003;116(Pt 3):499–511.
10. Bornstein S, White R, et al. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. J Clin Invest. 2009;119(11):3408–19.
11. Brabletz T, Jung A, et al. Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer. 2005;5(9):744–9.
12. Bracken CP, Gregory PA, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 2008;68(19):7846–54.
13. Calone I, Souchelnytskyi S. Inhibition of TGFbeta signaling and its implications in anticancer treatments. Exp Oncol. 2012;34(1):9–16.
14. Castanon I, Von Stetina S, et al. Dimerization partners determine the activity of the Twist bHLH protein during Drosophila mesoderm development. Development. 2001;128(16):3145–59.
15. Chen C, Wei Y, et al. Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. PLoS One. 2011;6(1):e16466.
16. Cheng GZ, Chan J, et al. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. Cancer Res. 2007;67(5):1979–87.
17. Childs G, Fazzari M, et al. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol. 2009;174(3):736–45.

18. Chu PY, Hu FW, et al. Epithelial-mesenchymal transition transcription factor ZEB1/ZEB2 co-expression predicts poor prognosis and maintains tumor-initiating properties in head and neck cancer. Oral Oncol. 2012.
19. Clarke MF, Dick JE, et al. Cancer stem cells–perspectives on current status and future directions: AACR Workshop on cancer stem cells.. Cancer Res. 2006;66(19):9339–44.
20. Comijn J, Berx G, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell. 2001;7(6):1267–78.
21. Dangi-Garimella S, Strouch MJ, et al. Collagen regulation of let-7 in pancreatic cancer involves TGF-beta1-mediated membrane type 1-matrix metalloproteinase expression. Oncogene. 2011;30(8):1002–8.
22. Davis BN, Hilyard AC, et al. Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. Mol Cell. 2010;39(3):373–84.
23. Davis SJ, Divi V, et al. Metastatic potential of cancer stem cells in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2010;136(12):1260–6.
24. Dennis M, Wang G, et al. Snail controls the mesenchymal phenotype and drives erlotinib resistance in oral epithelial and head and neck squamous cell carcinoma cells. Otolaryngol Head Neck Surg. 2012;147(4):726–32.
25. Escriva M, Peiro S, et al. Repression of PTEN phosphatase by Snail1 transcriptional factor during gamma radiation-induced apoptosis. Mol Cell Biol. 2008;28(5):1528–40.
26. Fendrich V, Waldmann J, et al. Unique expression pattern of the EMT markers Snail, Twist and E-cadherin in benign and malignant parathyroid neoplasia. Eur J Endocrinol. 2009;160(4):695–703.
27. Fukai Y, Fukuchi M, et al. Reduced expression of transforming growth factor-beta receptors is an unfavorable prognostic factor in human esophageal squamous cell carcinoma. Int J Cancer. 2003;104(2):161–6.
28. Fukuchi M, Masuda N, et al. Decreased Smad4 expression in the transforming growth factor-beta signaling pathway during progression of esophageal squamous cell carcinoma. Cancer. 2002;95(4):737–43.
29. Garzon R, Calin GA, et al. MicroRNAs in Cancer. Annu Rev Med. 2009;60:167–79.
30. Gasparotto D, Polesel J, et al. Overexpression of TWIST2 correlates with poor prognosis in head and neck squamous cell carcinomas. Oncotarget. 2011;2(12):1165–75.
31. Gill K, Mohanti BK, et al. Quantification of p38alphaMAP kinase: a prognostic marker in HNSCC with respect to radiation therapy. Clin Chim Acta. 2012;413(1–2):219–25.
32. Gordon KJ, Kirkbride KC, et al. Bone morphogenetic proteins induce pancreatic cancer cell invasiveness through a Smad1-dependent mechanism that involves matrix metalloproteinase-2. Carcinogenesis. 2009;30(2):238–48.
33. Gregory PA, Bert AG, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008;10(5):593–601.
34. Gregory PA, Bracken CP, et al. An autocrine TGF-beta/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. Mol Biol Cell. 2011;22(10):1686–98.
35. Haddad Y, Choi W, et al. Delta-crystallin enhancer binding factor 1 controls the epithelial to mesenchymal transition phenotype and resistance to the epidermal growth factor receptor inhibitor erlotinib in human head and neck squamous cell carcinoma lines. Clin Cancer Res. 2009;15(2):532–42.
36. Hamamori Y, Sartorelli V, et al. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell. 1999;96(3):405–13.
37. Han G, Lu SL, et al. Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. J Clin Invest. 2005;115(7):1714–23.
38. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004;5(7):522–31.
39. Hoot KE, Lighthall J, et al. Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. J Clin Invest. 2008;118(8):2722–32.

40. Hurd TW, Gao L, et al. Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. Nat Cell Biol. 2003;5(2):137–42.
41. Ikenouchi J, Matsuda M, et al. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci. 2003;116(Pt 10):1959–67.
42. Janda E, Lehmann K, et al. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol. 2002;156(2):299–313.
43. Kajita M, McClinic KN, et al. Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress. Mol Cell Biol. 2004;24(17):7559–66.
44. Katafiasz D, Smith LM, et al. Slug (SNAI2) expression in oral SCC cells results in altered cell-cell adhesion and increased motility. Cell Adh Migr. 2011;5(4):315–322.
45. Kojc N, Zidar N, et al. Expression of CD34, alpha-smooth muscle actin, and transforming growth factor beta1 in squamous intraepithelial lesions and squamous cell carcinoma of the larynx and hypopharynx. Hum Pathol. 2005;36(1):16–21.
46. Kong W, Yang H, et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol. 2008;28(22):6773–84.
47. Kwak PB, Iwasaki S, et al. The microRNA pathway and cancer. Cancer Sci. 2010;101(11): 2309–15.
48. Lee YM, Park T, et al. Twist-mediated activation of the NK-4 homeobox gene in the visceral mesoderm of Drosophila requires two distinct clusters of E-box regulatory elements. J Biol Chem. 1997;272(28):17531–41.
49. Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev. 2007;21(9):1025–30.
50. Levy L, Hill CS. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev. 2006;17(1–2):41–58.
51. Li AG, Lu SL, et al. Smad3 knockout mice exhibit a resistance to skin chemical carcinogenesis. Cancer Res. 2004;64(21):7836–45.
52. Li QQ, Xu JD, et al. Twist1-mediated adriamycin-induced epithelial-mesenchymal transition relates to multidrug resistance and invasive potential in breast cancer cells. Clin Cancer Res. 2009;15(8):2657–65.
53. Li Y, Yang J, et al. Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. J Clin Invest. 2003;112(4):503–16.
54. Lo WL, Yu CC, et al. MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells. J Pathol. 2011;223(4):482–95.
55. Lombaerts M, Wezel T van, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br J Cancer. 2006;94(5):661–71.
56. Lu SL, Reh D, et al. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. Cancer Res. 2004;64(13):4405–10.
57. Lu SL, Herrington H, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. Genes Dev. 2006;20(10):1331–42.
58. Mack B, Gires O. CD44s and CD44v6 expression in head and neck epithelia. PLoS One. 2008;3(10):e3360.
59. Maestro R, Dei Tos AP, et al. Twist is a potential oncogene that inhibits apoptosis.". Genes Dev. 1999;13(17):2207–17.
60. Mani SA, Guo W, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704–15.
61. Maseki S, Ijichi K, et al. Acquisition of EMT phenotype in the gefitinib-resistant cells of a head and neck squamous cell carcinoma cell line through Akt/GSK-3beta/snail signalling pathway. Br J Cancer. 2012;106(6):1196–204.
62. Massague J. TGFbeta in Cancer. Cell. 2008;134(2):215–30.

63. May CD, Sphyris N, et al. Epithelial-mesenchymal transition and cancer stem cells: a dangerously dynamic duo in breast cancer progression. Breast Cancer Res. 2011;13(1):202.
64. Mendelsohn AH, Lai CK, et al. Snail as a novel marker for regional metastasis in head and neck squamous cell carcinoma. Am J Otolaryngol. 2012;33(1):6–13.
65. Miettinen PJ, Ebner R, et al. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol. 1994;127(6 Pt 2):2021–36.
66. Moustakas A, Heldin CH. The regulation of TGFbeta signal transduction. Development. 2009;136(22):3699–714.
67. Muro-Cacho CA, Rosario-Ortiz K, et al. Defective transforming growth factor beta signaling pathway in head and neck squamous cell carcinoma as evidenced by the lack of expression of activated Smad2. Clin Cancer Res. 2001;7(6):1618–26.
68. Natsugoe S, Xiangming C, et al. Smad4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. Clin Cancer Res. 2002;8(6): 1838–42.
69. Nelson KM, Weiss GJ. MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther. 2008;7(12):3655–60.
70. Nguyen PT, Kudo Y, et al. N-cadherin expression is involved in malignant behavior of head and neck cancer in relation to epithelial-mesenchymal transition. Histol Histopathol. 2011;26(2):147–56.
71. Nieto MA. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol. 2002;3(3):155–66.
72. Nijkamp MM, Span PN, et al. Expression of E-cadherin and vimentin correlates with metastasis formation in head and neck squamous cell carcinoma patients. Radiother Oncol. 2011;99(3):344–8.
73. Oh SY, Kang HJ, et al. CD44-negative cells in head and neck squamous carcinoma also have stem-cell like traits. Eur J Cancer. 2012.
74. Ohkubo T, Ozawa M. The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. J Cell Sci. 2004;117(Pt 9):1675–85.
75. Ou DL, Chien HF, et al. Role of Twist in head and neck carcinoma with lymph node metastasis. Anticancer Res. 2008;28(2B):1355–9.
76. Ozdamar B, Bose R, et al. Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science. 2005;307(5715):1603–9.
77. Pan D, Fujimoto M, et al. Twist-1 is a PPARdelta-inducible, negative-feedback regulator of PGC-1alpha in brown fat metabolism. Cell. 2009;137(1):73–86.
78. Park SM, Gaur AB, et al. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008;22(7): 894–907.
79. Peinado H, Olmeda D, et al. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer. 2007;7(6):415–28.
80. Petersen M, Pardali E, et al. Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene. 2010;29(9):1351–61.
81. Pries R, Witrkopf N, et al. Potential stem cell marker CD44 is constitutively expressed in permanent cell lines of head and neck cancer. Vivo. 2008;22(1):89–92.
82. Prince ME, Sivanandan R, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A. 2007;104(3):973–8.
83. Qiao B, Johnson NW, et al. Disclosure of a stem cell phenotype in an oral squamous cell carcinoma cell line induced by BMP-4 via an epithelial-mesenchymal transition. Oncol Rep. 2011;26(2):455–61.
84. Radisky DC, LaBarge MA. Epithelial-mesenchymal transition and the stem cell phenotype. Cell Stem Cell. 2008;2(6):511–2.
85. Ramdas L, Giri U, et al. miRNA expression profiles in head and neck squamous cell carcinoma and adjacent normal tissue. Head Neck. 2009;31(5):642–54.

86. Rogers SJ, Harrington KJ, et al. Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer Metastasis Rev. 2005;24(1):47–69.
87. Sakamoto K, Imanishi Y, et al. Overexpression of SIP1 and downregulation of E-cadherin predict delayed neck metastasis in stage I/II oral tongue squamous cell carcinoma after partial glossectomy. Ann Surg Oncol. 2012;19(2):612–9.
88. Sharabi AB, Aldrich M, et al. Twist-2 controls myeloid lineage development and function. PLoS Biol. 2008;6(12):e316.
89. Shiota M, Izumi H, et al. Twist and p53 reciprocally regulate target genes via direct interaction. Oncogene. 2008;27(42):5543–53.
90. Shirakihara T, Saitoh M, et al. Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta. Mol Biol Cell. 2007;18(9):3533–3544.
91. Sinpitaksakul SN, Pimkhaokham A, et al. TGF-beta1 induced MMP-9 expression in HNSCC cell lines via Smad/MLCK pathway. Biochem Biophys Res Commun. 2008;371(4):713–8.
92. Solanas G, Porta-de-la-Riva M, et al. E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. J Cell Sci. 2008;121(Pt 13):2224–34.
93. Song J, Chang I, et al. Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling. PLoS One. 2010;5(7):e11456.
94. Sorrentino A, Thakur N, et al. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol. 2008;10(10):1199–207.
95. Spicer DB, Rhee J, et al. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science. 1996;272(5267):1476–80.
96. Stemmer V, Craene B de, et al. Snail promotes Wnt target gene expression and interacts with beta-catenin. Oncogene. 2008;27(37):5075–80.
97. Stransky N, Egloff AM, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60.
98. Strutz F, Zeisberg M, et al. Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. Kidney Int. 2002;61(5):1714–28.
99. Sun S, Wang Z. Head neck squamous cell carcinoma c-Met(+) cells display cancer stem cell properties and are responsible for cisplatin-resistance and metastasis. Int J Cancer. 2011;129(10):2337–48.
100. Takayama S, Hatori M, et al. Inhibition of TGF-beta1 suppresses motility and invasiveness of oral squamous cell carcinoma cell lines via modulation of integrins and down-regulation of matrix-metalloproteinases. Oncol Rep. 2009;21(1):205–10.
101. Tannehill-Gregg SH, Kusewitt DF, et al. The roles of Smad2 and Smad3 in the development of chemically induced skin tumors in mice. Vet Pathol. 2004;41(3):278–82.
102. Thiery JP, Acloque H, et al. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.
103. Thuault S, Tan EJ, et al. HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. J Biol Chem. 2008;283(48):33437–46.
104. Thuault S, Valcourt U, et al. Transforming growth factor-beta employs HMGA2 to elicit epithelial-mesenchymal transition. J Cell Biol. 2006;174(2):175–83.
105. Tran DD, Corsa CA, et al. Temporal and spatial cooperation of Snail1 and Twist1 during epithelial-mesenchymal transition predicts for human breast cancer recurrence. Mol Cancer Res. 2011;9(12):1644–57.
106. Usami Y, Satake S, et al. Snail-associated epithelial-mesenchymal transition promotes oesophageal squamous cell carcinoma motility and progression. J Pathol. 2008;215(3):330–9.
107. Vega S, Morales AV, et al. Snail blocks the cell cycle and confers resistance to cell death. Genes Dev. 2004;18(10):1131–43.
108. Verschueren K, Remacle JE, et al. SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. J Biol Chem. 1999;274(29):20489–98.
109. Vincent T, Neve EP, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. Nat Cell Biol. 2009;11(8):943–50.

110. Wang D, Song H, et al. Mutation and downregulation of the transforming growth factor beta type II receptor gene in primary squamous cell carcinomas of the head and neck. Carcinogenesis. 1997;18(11):2285–90.
111. Welch DR, Fabra A, et al. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc Natl Acad Sci U S A. 1990;87(19):7678–82.
112. Wushou A, Pan HY, et al. Correlation of increased twist with lymph node metastasis in patients with oral squamous cell carcinoma. J Oral Maxillofac Surg. 2012;70(6):1473–9.
113. Xie W, Aisner S, et al. Alterations of Smad expression and activation in defining 2 subtypes of human head and neck squamous cell carcinoma. Head Neck. 2013;35(1):76–85.
114. Xie W, Bharathy S, et al. Frequent alterations of Smad signaling in human head and neck squamous cell carcinomas: a tissue microarray analysis. Oncol Res. 2003;14(2):61–73.
115. Xu T, Yu CY, et al. Bone morphogenetic protein-4-induced epithelial-mesenchymal transition and invasiveness through Smad1-mediated signal pathway in squamous cell carcinoma of the head and neck. Arch Med Res. 2011;42(2):128–37.
116. Yamashita M, Fatyol K, et al. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. Mol Cell. 2008;31(6):918–24.
117. Yang J, Mani SA, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004;117(7):927–39.
118. Yang MH, Hsu DS, et al. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell Biol. 2010;12(10):982–92.
119. Yang MH, Wu MZ, et al. Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nat Cell Biol. 2008;10(3):295–305.
120. Yang MH, Chang SY, et al. Overexpression of NBS1 induces epithelial-mesenchymal transition and co-expression of NBS1 and Snail predicts metastasis of head and neck cancer. Oncogene. 2007;26(10):1459–67.
121. Yin Z, Xu XL, et al. Regulation of the twist target gene tinman by modular cis-regulatory elements during early mesoderm development. Development. 1997;124(24):4971–82.
122. Yokoyama K, Kamata N, et al. Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. Oral Oncol. 2001;37(1):65–71.
123. Yu L, Hebert MC, et al. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J. 2002;21(14):3749–59.
124. Zhang P, Zhang Y, et al. Side population in oral squamous cell carcinoma possesses tumor stem cell phenotypes. Cancer Lett. 2009;277(2):227–34.
125. Zubeldia IG, Bleau AM, et al. Epithelial to mesenchymal transition and cancer stem cell phenotypes leading to liver metastasis are abrogated by the novel TGFbeta1-targeting peptides P17 and P144. Exp Cell Res. 2013;319(3):12–22.
126. Zuo JH, Zhu W, et al. Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. J Cell Biochem. 2011;112(9):2508–17.

Chapter 10
The Wnt/β-catenin Signaling Circuitry in Head and Neck Cancer

Rogerio M. Castilho and J. Silvio Gutkind

Abstract We have recently gained an unprecedented knowledge of the most frequent genetic alterations in head and neck squamous cell carcinoma (HNSCC). We have also learned that the aberrant function of multiple signaling networks contributes to HNSCC initiation and progression, including the persistent activation of the PI3K/Akt/mTOR, NFκB, and STAT3 signaling pathways and decreased antiproliferative responses to Notch and TGF-β. Emerging evidence suggests that many regulatory components of the Wnt signaling pathway are also dysfunctional in HNSCC, leading to increased nuclear β-catenin levels. This includes increased expression of Wnt family members and their coreceptor, Frizzled, loss of heterozygosity of the adenomatosis polyposis coli (APC) tumor suppressor gene, and epigenetic events leading to decreased expression of APC and the Wnt antagonists the secreted Frizzled-related proteins (SFRPs), Wnt inhibitory factors (WIFs) and Dickkopf family members (DKKs), primarily by promoter hypermethylation. Wnt/β-catenin controls cell fate decisions in normal epithelial stem cells, but persistent β-catenin signaling contributes to increased growth, metastatic potential and resistance to chemotherapy in HNSCC and their tumor-initiating cells. While the role of Wnt/β-catenin in HNSCC is not as well defined as in other cancers, the development of small molecule inhibitors of the Wnt/β-catenin pathway for other cancer types may soon afford novel targeted options for the treatment of HNSCC lesions displaying aberrant Wnt signaling.

Keywords Wnt · Squamous carcinoma · Oral cancer · HPV · β-catenin · Epigenetics · Signal transduction · Oncogenome · Cancer stem cells

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10.1 Introduction

With more than 500,000 new cases annually, head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancers globally [1], which results in more than 11,000 deaths each year in the USA alone [1]. The 5-year survival of newly diagnosed HNSCC patients is approximately 50 % and has improved only marginally over the past three decades [2]. This may reflect the fact that most HNSCC cases are still diagnosed at advanced stages, when they fail to respond to the currently available therapeutic options. While the most common risk factors involved in HNSCC pathogenesis, including alcohol and tobacco usage, human papillomavirus (HPV) infection, and areca nut chewing are well recognized [3]–[5], we still have an incomplete understanding of the molecular mechanisms underlying this disease, thus preventing the development of new molecular-targeted treatment options and chemopreventive strategies.

Recent deep sequencing efforts have provided an unprecedented knowledge of the most frequent somatic genetic alterations in HNSCC [6], [7]. They include inactivating mutations in *Notch*, *p53*, and *p16^{ink4a}* tumor suppressor genes, in addition to non-overlapping activating mutations of the *PIK3CA* and *RAS* oncogenes and inactivation of the tumor suppressor gene *PTEN*. Parallel efforts have revealed that the aberrant function of multiple signaling networks contribute to HNSCC initiation and progression [8]. In this regard, our laboratory has focused on the study of the PI3K/Akt/mTOR signaling pathway, which is activated in the vast majority of the HNSCC cases [9], [10], and which functionally interacts with the Wnt/β-catenin pathway to regulate its activity. In this book chapter, we will focus on the contribution of the Wnt/β-catenin pathway to HNSCC, given its central role in the control of epithelial stem cell fate decisions, and the emerging evidence for the dysregulated expression and function of components of the Wnt/β-catenin signaling system in HNSCC initiation and progression.

10.2 The Wnt Signaling System

Wnt signaling (Fig. 10.1) is essential for embryogenesis and is required for the maintenance of multiple stem cell compartments in adults, and hence for tissue homeostasis and regeneration upon injury [11], [12]. During development, Wnt signaling is responsible for axial patterning, and regulates cell proliferation, migration, adhesion, and stem cell fate decisions in multiple tissues, including self-renewal and commitment to differentiation, as part of a signaling mechanism conserved from flies to humans [13]–[17]. This remarkable biological role in organismal development and adult tissue maintenance is possible due to the large variety of signaling outcomes resulting from the tissue-specific and temporally controlled expression of over 19 secreted Wnt cysteine-rich glycoproteins, which interact with multiple seven-span transmembrane receptors of the Frizzled family, and with two members of the low-density-lipoprotein receptor-related protein family, LRP5 or LRP6 [13], [16].

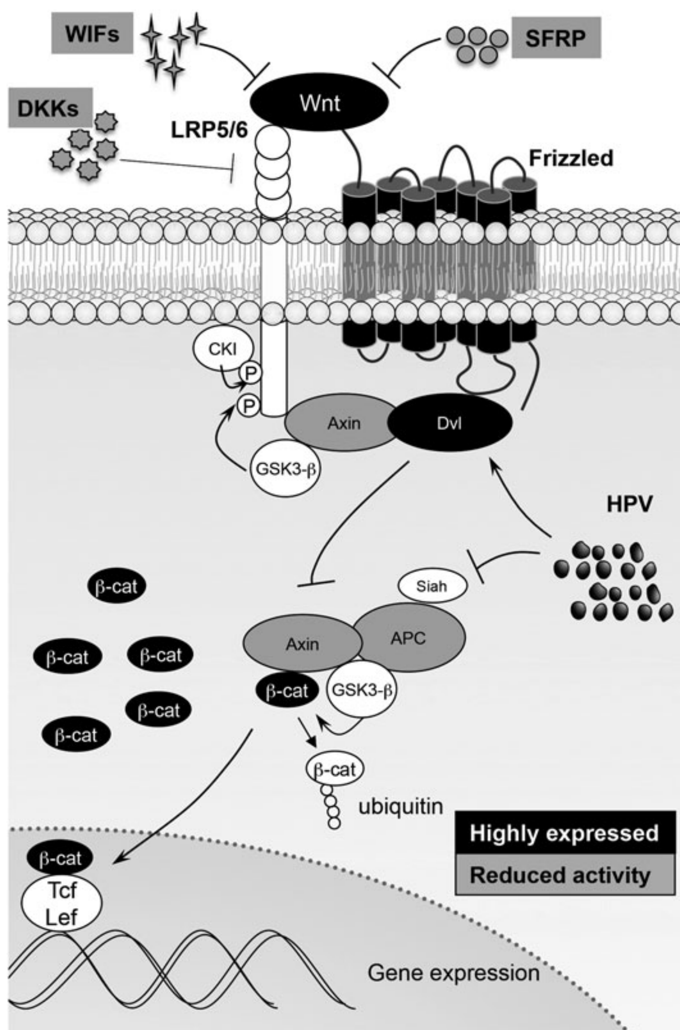

Fig. 10.1 Representation of known alterations in components of the Wnt/β-catenin signaling pathway in HNSCC. Molecules associated with β-catenin signaling are often highly expressed (*Black*), while those known to exert an inhibitory role are often underexpressed (*Gray*), due to genetic and epigenetic alterations. Infection of HPV-16 oncovirus results in activation of the Wnt/β-catenin pathway upon association of HPV E6 with Dvl-2 and by inhibiting the E3 ubiquitin-ligase protein Siah associated with the degradation of β-catenin. Aberrant changes in HNSCC and infection by HPV leads to the accumulation of free β-catenin that translocate to the nucleus activating the expression of Wnt/β-catenin target genes (see text for details)

Specific Wnt proteins can initiate the activation of several major signaling pathways: the "canonical" Wnt/β-catenin pathway, discussed at length in this chapter, and the "non-canonical" Wnt pathways, which include the planar cell polarity (PCP), c-Jun amino-terminal kinase (JNK), Rho, and calcium signaling pathways (reviewed in [16]

and [17], and not discussed further in this chapter). Among them, the best understood is canonical β-catenin-mediated Wnt signaling, altered function of which is associated with several biological processes, including development and cancer progression. Indeed, activation of the Wnt/β-catenin pathway is a frequent event in colon, kidney, prostate, and thyroid cancer and melanoma, among others [18], including tumors arising from the mammary gland and HNSCC (reviewed in [19], [20], [21]).

In the absence of Wnt stimulation, β-catenin binds a destruction complex that is composed of Axin, a scaffold protein that forms a large molecular complex with the tumor suppressor protein adenomatosis polyposis coli (APC), casein kinase Iα (CKIα), and the kinase GSK-3β. GSK-3β is responsible for the phosphorylation of β-catenin, thereby marking it for its subsequent ubiquitin-dependent degradation in the proteosome [16], [22]. Binding of Wnt to Frizzled and LRP5/6 results in the rapid phosphorylation of LRP5/6, which limits the function of the destruction complex. This leads to the accumulation of newly synthesized unphosphorylated β-catenin, which subsequently translocates to the nucleus where it complexes with T cell factor (TCF) and lymphoid enhancer-binding factor (LEF) transcription factors, thereby regulating gene expression [23]. In addition to Frizzled and LRP5/6, Wnt can bind to and initiate signaling from multiple other cell surface molecules, including *r*eceptor tyrosine kinase-like *o*rphan *r*eceptor (ROR), *p*rotein tyrosine (*Y*) *k*inase 7 (PTK7), *r*eceptor tyrosine (*Y*) *k*inase (RYK) family proteins, *mu*scle *s*keletal receptor tyrosine *k*inase (MUSK), and even some proteoglycan families, whose roles during development and cancer progression are just beginning to be appreciated [24]. For the purpose of this chapter, we will focus primarily in the canonical Wnt/β-catenin pathway given its best-characterized role in epithelial stem cell biology and HNSCC disease progression.

10.3 Wnt Signaling at the Cell Membrane

Most of the current work on Wnt signaling addresses the intracellular signaling pathway associated with the dynamics of β-catenin expression and localization, and its structural roles and downstream transcriptional targets. However, Wnt and its multiple regulatory proteins and receptors are capable of contributing to a multitude of tissue-specific biological functions and signaling mechanisms. For the purpose of describing Wnt function in more detail, we will focus on the activation of Wnt signaling in the epidermis. Wnt expression in the skin is initially observed in the dermis adjacent to the epidermis, where it plays a fundamental role in the formation of placodes, that are formed by the accumulation of Wnt-responsive epithelial cells [25] (reviewed in [26]–[28]). A multistep molecular cross-talk is then initiated between the dermal and epidermal compartment, involving developmental and morphogenic gene-expression programs, that ultimately result in the formation of the hair follicles [24]. Once fully developed, the hair follicles retain detectable nuclear levels of β-catenin in their epidermal (precortex and matrix cells) and dermal (dermal papilla) components, which are essential for the long-term maintenance of the hair

follicle structures [24], [29]. Of interest, Wnt and β-catenin functions are not always equivalent in this differentiation pathway. Genetic mutations of β-catenin resulting in its stabilization and hence, persistent signaling in gene transcription can result in a completely different phenotype than the constitutive activation of Wnt signaling at the cell membrane level. While uncontrolled accumulation of the active form of β-catenin in the hair follicles and skin results in tumor development [30] and presumably the expansion of the epithelial stem cell compartment, as observed in other systems [31], Wnt overexpression triggers epidermal and stem cell differentiation and senescence, with progression to complete depletion of hair follicle structures [32]. Such discrepancy is likely associated with the emerging complexity of the Wnt-induced pathways [33], [34], suggesting fundamental differences in the pathobiology of tumor formation mediated by components of the Wnt pathway localized at the membrane and cytosol, versus the sole accumulation of β-catenin in the nucleus.

For example, Frizzled-1, a Wnt coreceptor located at the plasma membrane (Fig. 10.1), and potentially a G-protein-linked receptor candidate, can induce the differentiation of mouse teratocarcinoma cells resulting in the formation of primitive endoderm upon expression of Wnt-8 [35] (reviewed in [36]). Wnt signaling at the plasma membrane has been also shown to play a fundamental role in the protection of cardiac stem cells from apoptosis induced by H_2O_2 [37], while loss of function of Wnt-1 is associated with the autosomal-recessive osteogenesis imperfecta [38] that is characterized by impaired osteoblast differentiation. Similarly, homozygous mutation of LRP5 and consequence disruption of the canonical Wnt signaling results in recessive osteoporosis-pseudoglioma syndrome, whereas Wnt gain-of-function enhances osteoblast differentiation and consequently augments bone density [39]. Similar findings were observed regarding the induction of differentiation of mesenchymal stem cells into retinal neuron-like cells displaying retinal neuron-like markers [40] and in Wnt-1-induced differentiation during midbrain development [41]. Overall, the molecular signaling events resulting from Wnt pathway activation at the cell membrane level is often associated with tissue maintenance by the activation of gene expression signatures in stem cells and their differentiated progeny, and their deficiency is associated with neurodegenerative diseases (reviewed in [42]), cardiovascular diseases [43]–[45], and bone homeostasis defects [38], [46]. However, cancer-associated mutations in the Wnt signaling system rarely involve Wnt and its receptors and coreceptors at the plasma membrane, suggesting a more relevant role for the nuclear functions of Wnt, primarily mediated through β-catenin, in tumor progression.

10.4 Wnt Signaling at the Transcriptional Level

Many proliferative lesions are associated with the aberrant function of intracellular Wnt signaling components, which ultimately results in the cytoplasmic accumulation of β-catenin and its translocation to the nucleus. Most of them involve loss-of-function mutations in components of the destruction complex or nonoverlapping gain-of-function mutations in β-catenin. A well-defined example of such mutations

include those associated with familial adenomatous polyposis (FAP), a relatively rare inherited disorder characterized by the development of multiple benign polyps in the colon in early adulthood, which can evolve into colon carcinomas [47], [48]. This disease results from the loss-of-function mutations and gene deletion of the APC gene, a central member of the destruction complex [49]. APC acts as a tumor suppressor protein responsible for the destabilization of free β-catenin (also known as CTNNB1). While APC can also perform additional biological roles, the direct association between Wnt signaling and colon cancer is further strengthened by the fact that nonoverlapping mutations in both APC and CTNNB1 are frequently found in nonfamilial adenomatous polyposis tumors [50]–[52].

Structural alterations in other components of the β-catenin destruction machinery are also associated with tumor development and progression. Axin, for example, destabilizes β-catenin upon binding APC through its RGS domain [53]. Mutations in the Axin 1- and 2-encoding genes are frequent events in human hepatocellular carcinomas (HCCs), which also present mutations in β-catenin and *Tcf1* genes [54]. The emerging concept that *AXIN1* is a tumor suppressor gene is based on loss of heterozygosity (LOH) found in poorly differentiated HCCs presenting unstable chromatin [55]. Finally, APC and Axin form the destruction complex by interacting with and modulating the serine/threonine kinase GSK3-β, which is ultimately responsible for β-catenin phosphorylation. The efficiency of GSK3-β in phosphorylating β-catenin is exponentially enhanced in the presence of Axin [22] and mutations in the Axin/GSK3-β binding interface are often observed in colon, lung, ovary, pancreas, rectum, breast, and hepatocellular carcinomas [56]–[58].

Two other components of the destruction complex are the proteins Disheveled (Dvl) and PP2A. Both proteins are essential for the proper functioning of the β-catenin regulatory complex, with Wnt signaling promoting the phosphorylation of Dvl, resulting in the inhibition of GSK3-β, accumulation of β-catenin, and the transcription of its target genes [18], [59]. At the protein level, Dvl proteins are highly expressed in non-small cell lung cancer (NSCLC), with the expression of Dvl-1 and Dvl-2 associated with advanced stages while Dvl-1 and Dvl-3 expression levels correlating with local lymph node metastasis [60]. The recent observation that histone deacetylases (HDACs) associate with and modulate Dvl proteins provided a novel mechanism by which cytosolic HDACs can control Wnt signaling. Specifically, the HDAC SIRT1 is capable of promoting constitutive Wnt signaling by forming a complex with Dvl proteins resulting in the regulation of β-catenin [61]. The emerging evidence that HDACs control Wnt signaling suggests that this signaling pathway can be regulated by molecules often involved in epigenetic regulation, which plays a fundamental role during embryogenesis, cancer promotion, and in the aggressive behavior of HNSCCs [62].

10.5 Wnt and Epigenetics

Understanding the mechanisms of epigenetic regulation of gene expression represents a unique opportunity to elucidate how cells can change the repertoire of molecules expressed, and hence their phenotype during embryogenesis and tumor

progression, independently from alterations in the DNA sequences [63], [64]. Epigenetic mechanisms contribute to the control of normal tissue homeostasis through the establishment and maintenance of tissue-specific gene expression signatures [63]. However, deregulated environmental cues can induce cellular transformation by altering local signaling circuitry, resulting in a dysfunctional epigenetic equilibrium. Under the influence of the microenvironment in which the tumors reside, cancer cells undergo progressive changes in gene expression programs resulting in distinct cellular behaviors and phenotypes, including increased tumor aggressiveness or induction of tumor quiescence.

The broad role of the Wnt signaling network during embryogenesis and tumor progression makes this pathway suitable for constant epigenetic modulation throughout the life span of an organism. This influence of epigenetics on components of the Wnt pathway may explain the relative low frequency of Wnt-associated mutations found in HNSCC (see below). Indeed, next generation sequencing of HNSCCs has only identified a modest number of mutations in the Wnt signaling pathway [6], [7] probably due the fact that many genes, especially tumor suppressor genes, are often found silenced in HNSCC. Similarly, constitutive activation of Wnt signaling in breast cancer is often associated with the silencing of the DACT1 gene (dapper, antagonist of beta-catenin, homolog 1), an antagonist of the Wnt/β-catenin responsible for reducing β-catenin activity [65]. DACT1 downregulation in breast cancer has been associated with its promoter methylation [65]. Similarly, promoter methylation of DACT2 results in loss or reduced antitumoral function and is found in more than half of the cases of hepatocellular carcinomas [66]. Epigenetic modulation of the Wnt signaling pathway has also been observed in esophageal squamous cell carcinoma. Deactivation of the secreted Frizzled-related protein 1 (SFRP1) gene, a Wnt inhibitor, is found in 95 % of the esophageal squamous cell carcinoma, where the SFRP1 promoter is methylated. Downregulation of SFRP1 results in its reduced inhibitory activity over the Wnt signaling pathway by allowing secreted Wnt to interact with the Frizzled receptor and consequently initiate signal transduction [67].

Interestingly, esophageal squamous cell carcinomas seem to have a particular predilection for loss of function of tumor suppressor genes that ultimately affect the Wnt/β-catenin signaling. Along with loss of SFRP1 function, Wnt-5A, which can activate noncanonical Wnt signaling, is also found frequently silenced through promoter CpG methylation, resulting in the activation of the canonical Wnt signaling pathway and increased levels of nuclear β-catenin [68]. Although the impact of epigenetic modifications has gained substantial attention in recent years, there are only a limited number of studies in the upper gastrointestinal tract and even fewer focused on HNSCC despite the growing evidence supporting the aberrant activation of the Wnt signaling pathway mediated by epigenetic alterations. One of the few examples is the identification of gene silencing induced by promoter methylation of the Wnt inhibitory factor-1 (WIF1), as shown in a large collection of primary tumors of nasopharyngeal squamous cell carcinomas and esophageal squamous cell carcinomas [69]. Interestingly, WIF1 promoter methylation is by far more prevalent in nasopharyngeal squamous cell carcinomas with 85 % of the cases presenting promoter methylation compared to only 27 % of the samples in esophageal squamous cell carcinoma cohorts [69].

10.6 Wnt and the Human Papillomavirus (HPV)

The epidemiology of HPV is associated with the transformation and progression of many human tumors including cervical cancer and HNSCC [10], [70], [71], [72]. HPV infection is the main cause of cervical cancers and later was established as responsible for a subpopulation of oral cancers, specifically associated with the oropharyngeal anatomical area, for reasons not well understood. Although the clinical progression of HPV-associated cervical cancer and HNSCC are quite distinct, both tumors share a similar degree of genetic and epigenetic alterations, suggesting a related transformation signature [9], [10], [73]. Indeed, both tumors present similarities such as the higher prevalence of HPV type 16 infections. Patients presenting HNSCC positive for HPV are of particular interest due to its unique clinical and tumor behavior characteristics compared to the known carcinogenesis progression associated with smoking and alcohol consumption.

Although the molecular mechanisms involved in tumor initiation and progression of HNSCCs associated with HPV infection are still not fully defined, there is a growing list of signaling pathways found to interact with the HPV carcinogenesis process. Among them, the Wnt/β-catenin signaling pathway has been shown to respond to the viral oncoproteins E6/E7 expression during normal infection and malignant conversion [74], [75]. Such mechanism involves the translocation of nuclear β-catenin induced by E6/E7 responsible for downregulating the E3 ubiquitin-ligase protein Siah, which is associated with the degradation of β-catenin independent of GSK3-β [75]. The molecular correlation between Wnt/β-catenin and HPV16 oncoproteins was further explored in a genetically defined animal model expressing full-length E6 oncoprotein targeted to the epidermis. Expression of E6 oncoprotein is sufficient to stimulate the Wnt signaling pathway *in vivo*, as judged by the nuclear accumulation of β-catenin [74]. The oncovirus HPV16 was also found associated with Dvl-2, known to play a role in the canonical and noncanonical Wnt signaling. Overall, it has been postulated that overexpression of the Wnt/β-catenin signaling pathway mediated by the HPV oncoproteins confers a "second hit" in the multistep carcinogenesis process, supporting a synergistic role of Wnt signaling in tumors associated with viral infection [76].

10.7 Wnt and Non-HPV-Associated Head and Neck Cancer

Whereas the involvement of the Wnt/β-catenin pathway is frequently observed at distinct stages of tumor initiation, progression, and metastasis, and it is often associated with the maintenance of cancer stem cells in several tumor types, including colorectal cancer [77], [78], NSCLC [79], and cancers arising in the breast [80] (reviewed in [81]), and prostate [82], among others; the precise role of Wnt signaling in the biology of HNSCC is still poorly defined. However, our mechanistic understanding of the contribution of Wnt signaling during the development of HNSCC is advancing. We now know that the regulatory components of Wnt signaling are often dysfunctional

in HNSCC, leading to increased pathway activity and overall increased accumulation of nuclear β-catenin [20], [21]. The APC tumor suppressor gene is often compromised in HNSCC, which is mainly driven by either LOH resulting in reduced gene activity [83] or by epigenetic events leading to APC promoter hypermethylation [84]. In both cases, β-catenin becomes stable and free to activate Wnt target genes upon nuclear translocation. Indeed, increased evidence suggests that altered expression of β-catenin found in HNSCC [85], [86] is not mutation driven [87] but rather a consequence of the deregulated function of members of the destruction complex. Similar findings are also observed within the rare subpopulation of HNSCC tumor-initiating cells, also called cancer stem cells [88], which present abnormal activity of β-catenin [89]. The involvement of tumor-initiating cells presenting constitutive expression of Wnt/β-catenin signaling is further associated with increased metastatic potential, invasive behavior, and resistance to chemotherapy in HNSCC [89], similar to that observed in other tumors such as colon cancer [77], [90].

Altered levels of expressions of Wnt, its cell membrane receptors, and their associated intracellular proteins have been also implicated in the biology of HNSCC. For example, the mRNAs for the Frizzled receptor and Dvl were found to be highly expressed in gene array studies of HNSCC [20]. Secreted Wnt proteins such as Wnt-14 were also found present at high levels when HNSCC tissues were microdissected and analyzed by liquid chromatography–tandem mass spectrometry (MS/MS) [21]. Sensitivity is considered the main barrier in the detection of proteins expressed at low levels, especially those localized in the interstitial tissues upon secretion. The combination of microdissected tumor tissues with use of the MS/MS approach provides much more sensitive strategy capable of identifying secreted proteins that may be responsible for cell signaling through membrane receptors. The importance of increasing sensitivity to detect Wnt family members in HNSCC may have future implications for the understanding of tumor transformation, progression, and phenotypic changes including epithelial–mesenchymal transition [91] during local invasion.

An alternative to an increase in activation-associated proteins is the loss of inactivating mechanisms. The molecular events originating from the interaction of secreted Wnt proteins with their cellular receptors are tightly regulated by Wnt antagonists, which are composed mainly of SFRPs, WIFs, and DKK family members, which bind and inhibit Wnt and the Wnt LRP5/6 coreceptors (Fig. 10.1). Of interest, many of these endogenous Wnt antagonists are targeted for deactivation in HNSCC due to epigenetic changes, as silencing of these genes can lead to constitutive Wnt signaling in HNSCC. In fact, the promoters of SFRP1, SFRP2, SFRP4 and SFRP5, WIF-1, and DKK-3 are found hypermethylated in HNSCC, with a particularly high incidence of promoter methylation in SFRP family members that is observed in 30–40 % of the analyzed cases, some of which were associated with alcohol and tobacco consumption, while others were found associated with HPV infection [92], [93].

The importance of active Wnt signaling during HNSCC progression is underscored by the *in vitro* positive results observed during direct therapeutic targeting of the pathway. The use of antibodies against Wnt-1 or Wnt-10b resulted in the inhibition of the proliferative capacity of HNSCC cancer cells *in vitro* followed by reduced

activity of the transcription factor LEF/TCF, reduced cyclin D1 protein expression, and induction of apoptosis [94]. Similarly, treatment of HNSCC with the Wnt antagonist SFRP1 resulted in tumor growth inhibition. Yet, not all analyzed tumor cells respond favorably to Wnt-1 and SFRP1-targeted therapies, which suggests that at least a subpopulation of HNSCC may not require Wnt signaling to sustain growth. Alternative strategic approaches aiming at inhibiting Wnt signaling have been explored in the context of other tumors, which can now be applied to HNSCC. This is the case with FAP patients, where COX2 inhibitor and non-steroidal anti-inflammatory drugs (NSAIDs) were sufficient to reduce the rate of formation of polyps [95], [96]. Other pharmacological strategies include the inhibition of Wnt ligands, enhancing Axin stability mediated by novel small molecules, inhibition of β-catenin transcriptional activity through disruption of its interaction with LEF/TCF, development of Dvl inhibitors, blocking antibodies, among many other possible experimental strategies (reviewed in [97]).

Overall, the role of the Wnt signaling in HNSCC warrants further investigation. It is clear that the role of Wnt in HNSCC is not as well defined as in other cancer types, such as colorectal tumors. However, the development of HNSCC is associated with the activation of multiple signaling circuitries, with a particular high frequency of overexpression of epidermal growth factor receptor (EGFR), along with aberrant Notch, NFκB, STAT, TGFβ, and PI3K/mTOR signaling, and the silencing of tumor suppressor gene including *P53, PTEN,* $p16^{ink4A}$ and $p14^{ARF}$, as discussed in other chapters in this book. The blueprint of the HNSCC oncogenome is therefore far more intricate than expected, as we have recently learned from next generation sequencing of a large collection of HNSCC specimens [7], [6] citation was added by the editor. Given the remarkable emerging molecular and cellular heterogeneity often observed in HNSCC, the application of multimodal therapeutic strategies are more likely to succeed upon the analysis of the genetic background of each HNSCC lesion. Indeed, the understanding of the molecular signaling networks governing tumor progression and metastatic spread in each individual HNSCC patient may reveal poorly explored actionable therapeutic mechanisms. We can expect that the current emphasis, on the development of small molecule inhibitors for the Wnt/β-catenin pathway for other cancer types, may soon afford novel pharmacological targeted options for the treatment of HNSCC lesions displaying aberrant Wnt signaling.

References

1. Conway DI, Hashibe M, Boffetta P, INHANCE consortium, Wunsch-Filho V, Muscat J, La Vecchia C, Winn DM. Enhancing epidemiologic research on head and neck cancer: INHANCE—The international head and neck cancer epidemiology consortium. Oral Oncol. 2009;45(9):743–6. doi:10.1016/j.oraloncology.2009.02.007.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics. CA: Cancer J Clinicians. 2008;58(2):71–96. doi:10.3322/CA.2007.0010.
3. Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. Cancer Cell. 2004;5(4):311–6.

4. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med. 2001;345(26):1890–900. doi:10.1056/NEJMra001375.
5. Bagan JV, Scully C. Recent advances in oral oncology 2007: epidemiology, aetiopathogenesis, diagnosis and prognostication. Oral Oncol. 2008;44(2):103–8. doi:10.1016/j.oraloncology.2008.01.008.
6. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs RA, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–7. doi:10.1126/science.1206923.
7. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60. doi:10.1126/science.1208130.
8. Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol. 2009;45(4–5):324–34. doi:10.1016/j.oraloncology.2008.07.011.
9. Molinolo AA, Marsh C, El Dinali M, Gangane N, Jennison K, Hewitt S, Patel V, Seiwert TY, Gutkind JS. mTOR as a molecular target in HPV-associated oral and cervical squamous carcinomas. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2012;18(9):2558–68. doi:10.1158/1078-0432.CCR-11-2824.
10. Gillison ML, Castellsague X, Chaturvedi A, Goodman MT, Snijders P, Tommasino M, Arbyn M, Franceschi S. Comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. Int J Cancer (Journal international du cancer). 2013. doi:10.1002/ijc.28201.
11. Wang J, Wynshaw-Boris A. The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. Curr Opin Genet Dev. 2004;14(5):533–9. doi:10.1016/j.gde.2004.07.013.
12. Kuhl SJ, Kuhl M. On the role of Wnt/beta-catenin signaling in stem cells. Biochimica et biophysica acta. 2013;1830(2):2297–306. doi:10.1016/j.bbagen.2012.08.010.
13. Nusse R. Wnt signaling and stem cell control. Cell Research. 2008;18(5):523–7. doi:10.1038/cr.2008.47.
14. Chang CH, Jiang TX, Lin CM, Burrus LW, Chuong CM, Widelitz R. Distinct Wnt members regulate the hierarchical morphogenesis of skin regions (spinal tract) and individual feathers. Mechanisms of development. 2004;121(2):157–71. doi:10.1016/j.mod.2003.12.004.
15. Olivera-Martinez I, Thelu J, Teillet MA, Dhouailly D. Dorsal dermis development depends on a signal from the dorsal neural tube, which can be substituted by Wnt-1. Mech Develop. 2001;100(2):233–44.
16. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling diseases and therapies. Nat Rev Genet. 2004;5(9):691–701. doi:10.1038/nrg1427.
17. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005;434(7035):843–50. doi:10.1038/nature03319.
18. Polakis P. Wnt signaling and cancer. Genes Dev. 2000;14(15):1837–51.
19. Lindvall C, Bu W, Williams BO, Li Y. Wnt signaling, stem cells, and the cellular origin of breast cancer. Stem Cell Rev. 2007;3(2):157–68.
20. Leethanakul C, Patel V, Gillespie J, Pallente M, Ensley JF, Koontongkaew S, Liotta LA, Emmert-Buck M, Gutkind JS. Distinct pattern of expression of differentiation and growth-related genes in squamous cell carcinomas of the head and neck revealed by the use of laser capture microdissection and cDNA arrays. Oncogene. 2000;19(28):3220–4. doi:10.1038/sj.onc.1203703.

21. Baker H, Patel V, Molinolo AA, Shillitoe EJ, Ensley JF, Yoo GH, Meneses-Garcia A, Myers JN, El-Naggar AK, Gutkind JS, Hancock WS. Proteome-wide analysis of head and neck squamous cell carcinomas using laser-capture microdissection and tandem mass spectrometry. Oral Oncol. 2005;41(2):183–99. doi:10.1016/j.oraloncology.2004.08.009.
22. Hart MJ, de los SR, Albert IN, Rubinfeld B, Polakis P. Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol: CB. 1998;8(10):573–81.
23. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science. 2004;303(5663):1483–7. doi:10.1126/science.1094291.
24. Niehrs C. The complex world of WNT receptor signalling. Nat Rev Mol Cell Biol. 2012;13(12):767–79. doi:10.1038/nrm3470.
25. Andl T, Reddy ST, Gaddapara T, Millar SE. WNT signals are required for the initiation of hair follicle development. Dev Cell. 2002;2(5):643–53.
26. Alonso L, Fuchs E. Stem cells in the skin: waste not, Wnt not. Genes Dev. 2003;17(10): 1189–1200. doi:10.1101/gad.1086903.
27. Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. Nature Rev Mol Cell Biol. 2009;10(3):207–17. doi:10.1038/nrm2636.
28. Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, Hassan AB, Volpin D, Bressan GM, Piccolo S. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A. 2003;100(6):3299–304. doi:10.1073/pnas.0434590100.
29. Reddy S, Andl T, Bagasra A, Lu MM, Epstein DJ, Morrisey EE, Millar SE. Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. Mech Develop. 2001;107(1-2):69–82.
30. Gat U, DasGupta R, Degenstein L, Fuchs E. De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell. 1998;95(5):605–14.
31. Liu BY, McDermott SP, Khwaja SS, Alexander CM. The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A. 2004;101(12):4158–63. doi:10.1073/pnas.0400699101.
32. Castilho RM, Squarize CH, Chodosh LA, Williams BO, Gutkind JS. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. Cell Stem Cell. 2009;5(3):279–89. doi:10.1016/j.stem.2009.06.017.
33. Barker N, Bartfeld S, Clevers H. Tissue-resident adult stem cell populations of rapidly self-renewing organs. Cell Stem Cell. 2012;7(6):656–70. doi:10.1016/j.stem.2010.11.016.
34. Cruciat CM, Niehrs C. Secreted and transmembrane Wnt inhibitors and activators. Cold Spring Harb Perspect Med. 2013;3(3):a015081.
35. Liu T, Liu X, Wang H, Moon RT, Malbon CC. Activation of rat frizzled-1 promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via pathways that require Galpha(q) and Galpha(o) function. J Biol Chem. 1999;274(47):33539–44.
36. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. Nature Reviews Cancer. 2007;7(2):79–94. doi:10.1038/nrc2069.
37. Liu J, Wang Y, Du W, Liu W, Liu F, Zhang L, Zhang M, Hou M, Liu K, Zhang S, Yu B. Wnt1 inhibits hydrogen peroxide-induced apoptosis in mouse cardiac stem cells. PloS One. 2013;8(3):e58883. doi:10.1371/journal.pone.0058883.
38. Pyott SM, Tran TT, Leistritz DF, Pepin MG, Mendelsohn NJ, Temme RT, Fernandez BA, Elsayed SM, Elsobky E, Verma I, Nair S, Turner EH, Smith JD, Jarvik GP, Byers PH. WNT1 Mutations in Families Affected by Moderately Severe and Progressive Recessive Osteogenesis Imperfecta. Am J Hum Genet. 2013;92(4):590–7. doi:10.1016/j.ajhg.2013.02.009.
39. Ellies DL, Viviano B, McCarthy J, Rey JP, Itasaki N, Saunders S, Krumlauf R. Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. J Bone Miner Res. 2006;21(11):1738–49. doi:10.1359/jbmr.060810.
40. Xu Y, Gu Z, Shen B, Xu G, Zhou T, Jiang J, Xing J, Liu S, Li M, Tan W, Feng G, Sang A, Li L. Roles of Wnt/beta-catenin signaling in retinal neuron-like differentiation of bone marrow mesenchymal stem cells from nonobese diabetic mice. J Mol Neurosci: MN. 2013;49(2):250–61. doi:10.1007/s12031-012-9917-z.

41. Moreno-Bravo JA, Martinez-Lopez JE, Puelles E. Mesencephalic neuronal populations: new insights on the ventral differentiation programs. Histol Histopathol. 2012;27(12):1529–38.
42. Marchetti B, L'Episcopo F, Morale MC, Tirolo C, Testa N, Caniglia S, Serapide MF, Pluchino S. Uncovering novel actors in astrocyte-neuron crosstalk in Parkinson's disease: the Wnt/beta-catenin signaling cascade as the common final pathway for neuroprotection and self-repair. Eur J Neurosci. 2013. doi:10.1111/ejn.12166.
43. van Gijn ME, Daemen MJ, Smits JF, Blankesteijn WM. The wnt-frizzled cascade in cardiovascular disease. Cardiovasc Res. 2002;55(1):16–24.
44. Mani A, Radhakrishnan J, Wang H, Mani A, Mani MA, Nelson-Williams C, Carew KS, Mane S, Najmabadi H, Wu D, Lifton RP. LRP6 mutation in a family with early coronary disease and metabolic risk factors. Science. 2007;315(5816):1278–82. doi:10.1126/science.1136370.
45. Tomaszewski M, Charchar FJ, Barnes T, Gawron-Kiszka M, Sedkowska A, Podolecka E, Kowalczyk J, Rathbone W, Kalarus Z, Grzeszczak W, Goodall AH, Samani NJ, Zukowska-Szczechowska E. A common variant in low-density lipoprotein receptor-related protein 6 gene (LRP6) is associated with LDL-cholesterol. Arterioscl Throm Vas Biol. 2009;29(9):1316–21. doi:10.1161/ATVBAHA.109.185355.
46. Valero C, Zarrabeitia MT, Hernandez JL, Pineda B, Cano A, Garcia-Perez MA, Riancho JA. Relationship of sclerostin and secreted frizzled protein polymorphisms with bone mineral density: an association study with replication in postmenopausal women. Menopause. 2011;18(7):802–7. doi:10.1097/gme.0b013e3182091664.
47. Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D, et al. Identification of FAP locus genes from chromosome 5q21. Science. 1991;253(5020):661–5.
48. Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science. 1991;253(5020):665–9.
49. Roberts DM, Pronobis MI, Poulton JS, Waldmann JD, Stephenson EM, Hanna S, Peifer M. Deconstructing the sscatenin destruction complex: mechanistic roles for the tumor suppressor APC in regulating Wnt signaling. Mol Biol Cell. 2011;22(11):1845–63. doi:10.1091/mbc.E10-11-0871.
50. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell. 1996;87(2):159–70.
51. Segditsas S, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene. 2006;25(57):7531–7. doi:10.1038/sj.onc.1210059.
52. Chan TA, Wang Z, Dang LH, Vogelstein B, Kinzler KW. Targeted inactivation of CTNNB1 reveals unexpected effects of beta-catenin mutation. Proc Natl Acad Sci U S A. 2002;99(12):8265–8270. doi:10.1073/pnas.082240999.
53. Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, Kikuchi A. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem. 1998;273(18):10823–6.
54. Taniguchi K, Roberts LR, Aderca IN, Dong X, Qian C, Murphy LM, Nagorney DM, Burgart LJ, Roche PC, Smith DI, Ross JA, Liu W. Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene. 2002;21(31):4863–71. doi:10.1038/sj.onc.1205591.
55. Laurent-Puig P, Zucman-Rossi J. Genetics of hepatocellular tumors. Oncogene. 2006;25(27):3778–86. doi:10.1038/sj.onc.1209547.
56. Jin LH, Shao QJ, Luo W, Ye ZY, Li Q, Lin SC. Detection of point mutations of the Axin1 gene in colorectal cancers. Int J Cancer (Journal international du cancer). 2003;107(5):696–9. doi:10.1002/ijc.11435.
57. Webster MT, Rozycka M, Sara E, Davis E, Smalley M, Young N, Dale TC, Wooster R. Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomes Cancer. 2000;28(4):443–53.

58. Park JY, Park WS, Nam SW, Kim SY, Lee SH, Yoo NJ, Lee JY, Park CK. Mutations of beta-catenin and AXIN I genes are a late event in human hepatocellular carcinogenesis. Liver international : official journal of the International Association for the Study of the. Liver. 2005;25(1):70–6. doi:10.1111/j.1478-3231.2004.0995.x.
59. Ratcliffe MJ, Itoh K, Sokol SY. A positive role for the PP2A catalytic subunit in Wnt signal transduction. J Biol Chem 2000;275(46):35680–3. doi:10.1074/jbc.C000639200.
60. Wei Q, Zhao Y, Yang ZQ, Dong QZ, Dong XJ, Han Y, Zhao C, Wang EH. Dishevelled family proteins are expressed in non-small cell lung cancer and function differentially on tumor progression. Lung Cancer. 2008;62(2):181–92. doi:10.1016/j.lungcan.2008.06.018.
61. Holloway KR, Calhoun TN, Saxena M, Metoyer CF, Kandler EF, Rivera CA, Pruitt K. SIRT1 regulates Dishevelled proteins and promotes transient and constitutive Wnt signaling. Proc Natl Acad Sci U S A. 2010;107(20):9216–21. doi:10.1073/pnas.0911325107.
62. Giudice FS, Pinto DS Jr, Nor JE, Squarize CH, Castilho RM. Inhibition of histone deacetylase impacts cancer stem cells and induces epithelial-mesenchyme transition of head and neck cancer. PloS One. 2013;8(3):e58672. doi:10.1371/journal.pone.0058672.
63. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis. 2010;31(1):27–36. doi:10.1093/carcin/bgp220.
64. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nature Rev Genet. 2002;3(6):415–28. doi:10.1038/nrg816.
65. Yin X, Xiang T, Li L, Su X, Shu X, Luo X, Huang J, Yuan Y, Peng W, Oberst M, Kelly K, Ren G, Tao Q. DACT1, an antagonist to Wnt/beta-catenin signaling, suppresses tumor cell growth and is frequently silenced in breast cancer. Breast Cancer Res: BCR. 2013;15(2):R23. doi:10.1186/bcr3399.
66. Zhang X, Yang Y, Liu X, Herman JG, Brock MV, Licchesi JD, Yue W, Pei X, Guo M. Epigenetic regulation of the Wnt signaling inhibitor DACT2 in human hepatocellular carcinoma. Epigenetics. 2013;8(4). [Epub a head of print]
67. Meng Y, Wang QG, Wang JX, Zhu ST, Jiao Y, Li P, Zhang ST. Epigenetic inactivation of the SFRP1 gene in esophageal squamous cell carcinoma. Digest Dis Sci. 2011;56(11):3195–203. doi:10.1007/s10620-011-1734-7.
68. Li J, Ying J, Fan Y, Wu L, Ying Y, Chan AT, Srivastava G, Tao Q. WNT5A antagonizes WNT/beta-catenin signaling and is frequently silenced by promoter CpG methylation in esophageal squamous cell carcinoma. Cancer Biol Ther. 2010;10(6):617–24.
69. Chan SL, Cui Y, van Hasselt A, Li H, Srivastava G, Jin H, Ng KM, Wang Y, Lee KY, Tsao GS, Zhong S, Robertson KD, Rha SY, Chan AT, Tao Q. The tumor suppressor Wnt inhibitory factor 1 is frequently methylated in nasopharyngeal and esophageal carcinomas. Lab Invest J Tech Meth Pathol. 2007;87(7):644–50. doi:10.1038/labinvest.3700547.
70. Huibregtse JM, Beaudenon SL. Mechanism of HPV E6 proteins in cellular transformation. Semin Cancer Biol. 1996;7(6):317–26. doi:10.1006/scbi.1996.0041.
71. Ciesielska U, Nowinska K, Podhorska-Okolow M, Dziegiel P. The role of human papillomavirus in the malignant transformation of cervix epithelial cells and the importance of vaccination against this virus. Adv Clin Expt Med. 2012;21(2):235–44.
72. Rautava J, Syrjanen S. Biology of human papillomavirus infections in head and neck carcinogenesis. Head Neck Pathol. 2012;6(Suppl 1):S3-15. doi:10.1007/s12105-012-0367-2.
73. Madsen BE, Ramos EM, Boulard M, Duda K, Overgaard J, Nordsmark M, Wiuf C, Hansen LL. Germline mutation in RNASEL predicts increased risk of head and neck, uterine cervix and breast cancer. PloS One. 2008;3(6):e2492. doi:10.1371/journal.pone.0002492.
74. Bonilla-Delgado J, Bulut G, Liu X, Cortes-Malagon EM, Schlegel R, Flores-Maldonado C, Contreras RG, Chung SH, Lambert PF, Uren A, Gariglio P. The E6 oncoprotein from HPV16 enhances the canonical Wnt/beta-catenin pathway in skin epidermis in vivo. Mol Cancer Res: MCR. 2012;10(2):250–8. doi:10.1158/1541-7786.MCR-11-0287.
75. Rampias T, Boutati E, Pectasides E, Sasaki C, Kountourakis P, Weinberger P, Psyrri A. Activation of Wnt signaling pathway by human papillomavirus E6 and E7 oncogenes in HPV16-positive oropharyngeal squamous carcinoma cells. Mol Cancer Res: MCR. 2010;8(3):433–43. doi:10.1158/1541-7786.MCR-09-0345.

76. Bulut G, Fallen S, Beauchamp EM, Drebing LE, Sun J, Berry DL, Kallakury B, Crum CP, Toretsky JA, Schlegel R, Uren A. Beta-catenin accelerates human papilloma virus type-16 mediated cervical carcinogenesis in transgenic mice. PloS One.2011;6(11):e27243. doi:10.1371/journal.pone.0027243.
77. de Sousa EM, Vermeulen L, Richel D, Medema JP. Targeting Wnt signaling in colon cancer stem cells. Clin Cancer Res. 2011;17(4):647–53. doi:10.1158/1078-0432.CCR-10-1204.
78. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nature Cell Biol. 2010;12(5):468–76. doi:10.1038/ncb2048.
79. Shapiro M, Akiri G, Chin C, Wisnivesky JP, Beasley MB, Weiser TS, Swanson SJ, Aaronson SA. Wnt pathway activation predicts increased risk of tumor recurrence in patients with stage I non-small cell lung cancer. Ann Surg. 2013;257(3):548–54. doi:10.1097/SLA.0b013e31826d81fd.
80. Matsuda Y, Schlange T, Oakeley EJ, Boulay A, Hynes NE. WNT signaling enhances breast cancer cell motility and blockade of the WNT pathway by sFRP1 suppresses MDA-MB-231 xenograft growth. Breast Cancer Res: BCR. 2009;11(3):R32. doi:10.1186/bcr2317.
81. Howe LR, Brown AM. Wnt signaling and breast cancer. Cancer Biol Ther. 2004;3(1):36–41.
82. Hall CL, Kang S, MacDougald OA, Keller ET. Role of Wnts in prostate cancer bone metastases. J Cell Biochem. 2006;97(4):661–72. doi:10.1002/jcb.20735.
83. Chang KW, Lin SC, Mangold KA, Jean MS, Yuan TC, Lin SN, Chang CS. Alterations of adenomatous polyposis Coli (APC) gene in oral squamous cell carcinoma. Int J Oral Max Surg. 2000;29(3):223–6.
84. Worsham MJ, Chen KM, Meduri V, Nygren AO, Errami A, Schouten JP, Benninger MS. Epigenetic events of disease progression in head and neck squamous cell carcinoma. Archiv Otolaryngol—Head Neck Surg. 2006;132(6):668–77. doi:10.1001/archotol.132.6.668.
85. Ueda G, Sunakawa H, Nakamori K, Shinya T, Tsuhako W, Tamura Y, Kosugi T, Sato N, Ogi K, Hiratsuka H. Aberrant expression of beta- and gamma-catenin is an independent prognostic marker in oral squamous cell carcinoma. Int J Oral Max Surg. 2006;35(4):356–61. doi:10.1016/j.ijom.2005.07.023.
86. Mahomed F, Altini M, Meer S. Altered E-cadherin/beta-catenin expression in oral squamous carcinoma with and without nodal metastasis. Oral Dis. 2007;13(4):386–92. doi:10.1111/j.1601-0825.2006.01295.x.
87. Lo Muzio L, Goteri G, Capretti R, Rubini C, Vinella A, Fumarulo R, Bianchi F, Mastrangelo F, Porfiri E, Mariggio MA. Beta-catenin gene analysis in oral squamous cell carcinoma. Int J Immunopath Ph. 2005;18(3 Suppl):33–8.
88. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A. 2007;104(3):973–978. doi:10.1073/pnas.0610117104.
89. Song J, Chang I, Chen Z, Kang M, Wang CY. Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling. PloS One. 2010;5(7):e11456. doi:10.1371/journal.pone.0011456.
90. de Sousa EMF, Colak S, Buikhuisen J, Koster J, Cameron K, de Jong JH, Tuynman JB, Prasetyanti PR, Fessler E, van denBSP, Rodermond H, Dekker E, van derLCM, Pals ST, van deVMJ, Versteeg R, Richel DJ, Vermeulen L, Medema JP. Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. Cell Stem Cell. 2011;9(5):476–85. doi:10.1016/j.stem.2011.10.008.
91. Uraguchi M, Morikawa M, Shirakawa M, Sanada K, Imai K. Activation of WNT family expression and signaling in squamous cell carcinomas of the oral cavity. J Dent Res. 2004;83(4):327–32.
92. Pannone G, Bufo P, Santoro A, Franco R, Aquino G, Longo F, Botti G, Serpico R, Cafarelli B, Abbruzzese A, Caraglia M, Papagerakis S, Lo Muzio L. WNT pathway in oral cancer: epigenetic inactivation of WNT-inhibitors. Oncol Rep. 2010;24(4):1035–41.

93. Marsit CJ, McClean MD, Furniss CS, Kelsey KT (2006) Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. Int J Cancer (Journal international du cancer). 119(8):1761–6. doi:10.1002/ijc.22051.
94. Rhee CS, Sen M, Lu D, Wu C, Leoni L, Rubin J, Corr M, Carson DA. Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. Oncogene. 2002;21(43):6598–605. doi:10.1038/sj.onc.1205920.
95. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med. 1993;328(18):1313–6. doi:10.1056/NEJM199305063281805.
96. Phillips RK, Wallace MH, Lynch PM, Hawk E, Gordon GB, Saunders BP, Wakabayashi N, Shen Y, Zimmerman S, Godio L, Rodrigues-Bigas M, Su LK, Sherman J, Kelloff G, Levin B, Steinbach G, Group FAPS. A randomised, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatous polyposis. Gut. 2002;50(6):857–60.
97. Anastas JN, Moon RT. WNT signalling pathways as therapeutic targets in cancer. Nature Reviews Cancer. 2013;13(1):11–26. doi:10.1038/nrc3419.

Chapter 11
Human Papillomavirus (HPV)-Positive Head and Neck Cancer and the Wnt Signaling Pathway

Theodoros Rampias and Amanda Psyrri

Abstract All known human papillomaviruses (HPVs) are exclusively epitheliotropic. Upon entry into populations of stratified epithelial cells, the E6 and E7 oncoproteins encoded by high-risk HPV variants establish a productive infection by manipulating signaling processes in the host environment, leading ultimately to production of infectious particles in the upper epithelial layers. The mechanisms by which E6 and E7 promote cell-cycle progression and viral DNA replication are well established, and involve E6-dependent ubiquitination and degradation of the p53 tumor suppressor, and E7- and cullin 2-dependent ubiquitination and degradation of the retinoblastoma (Rb) tumor suppressor protein. Recent experimental work provides evidence that high-risk HPVs also manipulate the underlying differentiation status of cells by targeting the Wnt pathway to ensure progression of the viral replication cycle. This chapter summarizes the possible cell pathways involved in the activation of Wnt signaling in HPV-positive head and neck cancer.

Keywords HPV · Head and neck cancer · Wnt · β-Catenin

11.1 The HPV life cycle, epithelial differentiation, and the Wnt signaling pathway

As discussed in depth by Ragin (Chap. 3), a subset of squamous cell carcinomas of the head and neck (SCCHNs) has been found to arise as a consequence of infection with human papillomaviruses (HPVs). HPVs infect stratified populations of epithelial cells in the oropharynx and depend on epithelial differentiation for completion of their life cycle. Typically, oropharyngeal keratinocytes undergo a differentiation

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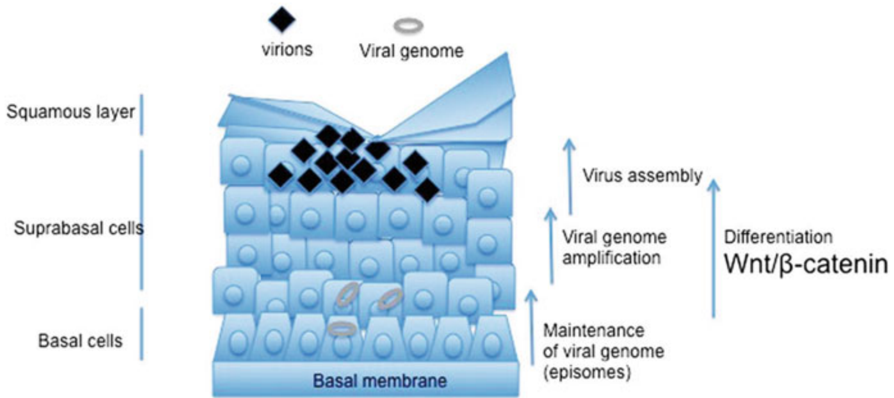
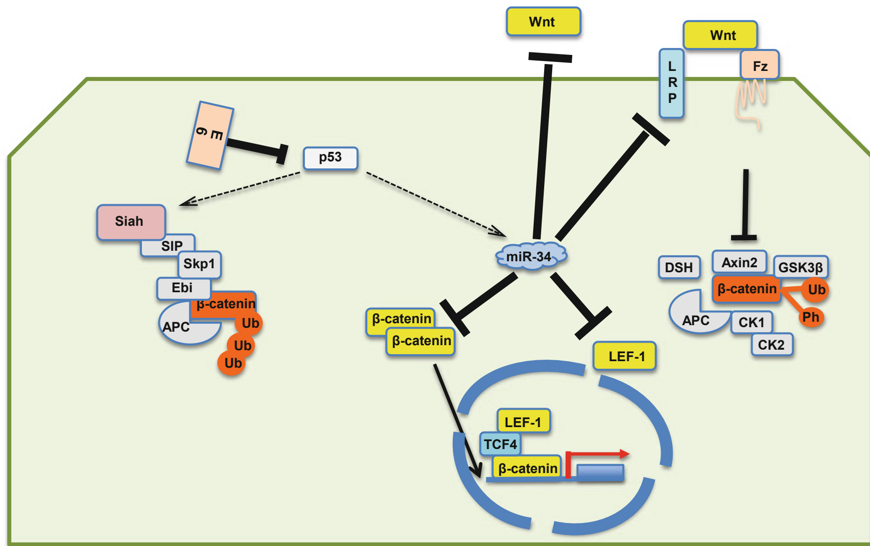


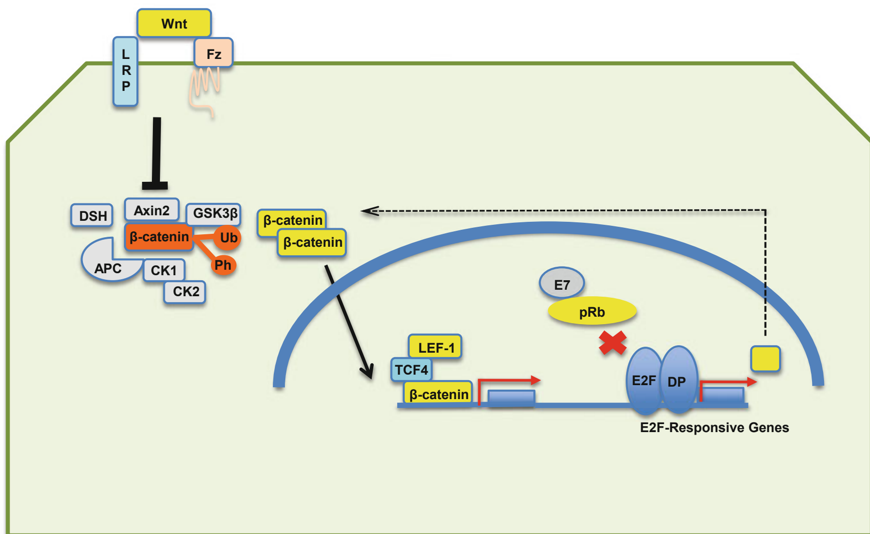
Fig. 11.1 HPV infection in head and neck epithelia. Active infection drives infected cells in the basal layer into proliferation, allowing viral genome amplification. Migration of cells into the upper layers is associated with activation of the Wnt signaling pathway, cell differentiation, and new virion production

process linked to cell migration from a basal layer through a suprabasal compartment until they form squamous, differentiated cells exposed to the environment. The expression of HPV-encoded genes is tightly regulated as the infected basal cell migrates towards the epithelial surface, and reciprocally influences cellular biology so as to enhance viral reproduction [1]–[4] (Fig. 11.1). Expression of E6 and E7 in the lower epithelial layers promotes the entry of keratinocytes into the S-phase, which creates an environment that favors viral genome replication and cell proliferation. Viral genome amplification, necessary for the production of infectious virions, is prevented until the levels of viral replication proteins rise, and depends on the co-expression of several viral proteins in the suprabasal layers. Virus capsid proteins are only expressed in infected cells that have migrated to the upper epithelial layers. The fact that HPV gene expression, replication, and persistence in the host are intimately linked to the differentiation state of the infected keratinocytes emphasizes the importance of the recent recognition that HPV can influence the status of signaling pathways that regulate cell fate commitment and differentiation.

Based on a model for HPV-induced carcinogenesis proposed by Gravitt [5], HPV infects basal epithelial cells at the sites of microtrauma. As many infected basal cells are induced to differentiate to fill the wound, these cells will produce an active papillomavirus infection. However, a few infected basal stem cells will remain undifferentiated, retain HPV in an episomal form, and slowly self-renew over long periods of time until triggered to differentiate by a new stimulus such as wound repair [5]. Recently, Tang and colleagues characterized an HPV-16,+ cancer stem cell-containing SCCHN cell line (UM-SCC-104) from a recurrent oral cavity tumor, providing further evidence for the existence of a subgroup of HPV-positive head and neck tumors that arise from HPV-infected stem cells [6]. This differentiation of latently infected stem cells, which is important to reactivate HPV-dependent gene transcription, requires the Wnt signaling pathway, and is thought to be crucial for the progression of HPV-induced carcinogenesis.



a



b

Fig. 11.2 **a** Altered regulation of Wnt signaling induced by HPV16 E6 mediated degradation of p53 in SCCHN. **b** Altered regulation of Wnt signaling induced by HPV16 E7 inactivation of pRb in SCCHN **KK**

The canonical Wnt signaling pathway (Fig. 11.2) is of central importance in regulating epithelium differentiation. Members of a family of extracellular Wnt proteins serve as ligands for cellular receptors including Frizzled and a co-receptor

such as low-density lipoprotein receptor-related protein (LRP). In the absence of Wnt ligands, free cytoplasmatic β-catenin is normally short-lived. It is recognized by a phosphorylation–destruction complex which consists of kinases and scaffold proteins, including glycogen synthase kinase-3β (GSK3β), casein kinases 1 and 2 (CK1 and CK2), Disheveled (DSH), adenomatous polyposis coli (APC), the F-box-containing ubiquitin ligase βTrCP, and Axin2. Following phosphorylation at serine and threonine residues, phosphorylated β-catenin is subsequently ubiquitinated and degraded [7].

Activation of Wnt signaling leads to the disassembly of the β-catenin degradation complex and inhibition of GSK3 activity. This process leads to the accumulation and nuclear translocation of β-catenin. Once it is translocated into the nucleus, β-catenin binds members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, of which TCF4 is the best characterized. β-Catenin/TCF4 complexes control the expression of several target genes that regulate cell polarity, proliferation, and differentiation including c-jun (JUN), c-myc (MYC), and cyclin D1 (CCND1) [8]–[12].

During embryonic development, Wnt-regulated β-catenin contributes to the establishment of the body axis and the orchestration of tissue and organ development [13]. Apart from its role in development, canonical Wnt signaling is involved in regulating cell proliferation, motility, survival, and stem cell maintenance in adult tissues. In the adult skin epithelium, stem cells have a higher pool of β-catenin available for Wnt signaling than their more differentiated progeny, and β-catenin activation can expand the stem cell pool [14]. This finding has subsequently been confirmed in experimental work on hematopoietic stem cells [15] and other tissues. In a study by Silva-Vargas and colleagues, a K14*Δ*Nβ-cateninER transgenic mouse (overexpressing a stabilized amino-terminally truncated β-catenin fused at the carboxyl terminus to the ligand-binding domain of a mutant estrogen receptor, under the control of K14 promoter) was used to study the activation of β-catenin signaling in adult mouse epidermis. In this model, conditional induction of β-catenin signaling activation in the basal layer of the epidermis by treatment with 4-hydroxy-tamoxifen (4OHT) promoted the expansion of the stem cell compartment of the hair follicles [16]. Other studies using different experimental strategies confirmed that β-catenin activation expands the stem cell compartment [17]–[20]. Affymetrix microarray analysis using RNA from the total skin of K14*Δ*Nβ-cateninER transgenic mouse treated for 7 days with 4OHT revealed that β-catenin activation upregulated the Sonic hedgehog (SHH) and Notch pathways, suggesting a mechanism for its activity in regulating differentiation. In this context, the mechanism by which HPV can manipulate WNT and potentially SHH and NOTCH have become topics of considerable interest.

11.2 Many viruses manipulate the Wnt signaling pathway

The manipulation of Wnt and Notch signaling pathways is a commonly employed strategy of viruses for achieving a cellular environment favorable for their replication. Strategies used by other classes of viruses to regulate Wnt signaling may

be informative to consider when analyzing the mechanisms employed by HPVs. As examples, tumorigenic gamma herpesviruses such as Kaposi's sarcoma associated herpesvirus (KSHV) and Epstein–Barr virus (EBV) activate the canonical Wnt/β–catenin pathway to induce cell proliferation. In case of KSHV, the expression of viral protein LANA (latency-associated nuclear protein) inhibits the β-catenin degradation machinery by binding to the GSK3β component of β-catenin destruction complex, sequestering GSK3β in the nucleus, thereby protecting β-catenin from ubiquitination [21]. EBV also induces β-catenin stabilization in infected B cells. In this case, the EBV oncoprotein LMP1 represses the expression of the human homolog of *Drosophila* seven in absentia (Siah-1), an E3 ubiquitin ligase that binds to antigen presenting cells (APC) and promotes the degradation of β-catenin in a GSK3β phosphorylation-independent manner [22].

Not all oncogenic viruses activate Wnt signaling. For example, other herpesviruses such as the human cytomegalovirus (HCMV) have been reported to inhibit the canonical Wnt signaling pathway, reducing the Wnt/β-catenin transcriptional activity in human dermal fibroblasts [23]. In HCMV-infected placenta cells, β-catenin is prevented from translocating in the nucleus, instead forming aggresomes in a discrete juxtanuclear location that are subsequently degraded [23]. The degree to which HPV induction of Wnt signaling is required for pathogenesis requires further testing.

11.3 HPV and stabilization of β-catenin in cervical and head and neck cancer

The association between oncogenic (high-risk) strains of HPV and cervical cancer is well established [24], and reflected by the high incidence of abnormally elevated nuclear accumulation of β-catenin in cervical cancer specimens [25]–[29]. High β-catenin levels are associated with advanced pathologic stages of adenocarcinoma [30]. Microarray expression studies conducted on cervical cancer tissues identified elevated expression of WNT GSK3β, and β-catenin [31], [32]. Van der Meide and colleagues determined that the promoters of nine negative regulators of the Wnt pathway (APC, AXIN2, DKK3, SFRP2, SFRP4, SFRP5, WIF1, and WNT5A) were hypermethylated in HPV-positive cervical cancer versus normal cervical tissue specimens, consistent with downregulated transcription, with the promoters of DKK3 and SFRP2 most specifically associated with the development of HPV-positive cervical adenocarcinoma [33].

HPV infection is also specifically associated with the activation of the Wnt pathway and elevation of β-catenin protein levels in SCCHN. High expression of the p16 protein is a reasonable surrogate marker for a clinically meaningful HPV infection in oropharyngeal squamous cell carcinoma (OSCC) [34], [35]. As pRb normally functions as a negative regulator of p16 expression, the functional inactivation of pRb by the HPV E7 protein results in a reciprocal overexpression of p16 protein in HPV-positive HNSCC. Loss of p16 expression by deletion, mutation, or hypermethylation is one of the most frequent genetic alterations in HPV16-negative HNSCC [36] and

therefore HPV-associated squamous cell carcinoma shows nuclear and cytoplasmic p16-overexpression, which is predominantly absent in HPV-negative squamous cell carcinoma. Weinberger and colleagues identified three tumor classes with distinct molecular and clinical features based on the detection of HPV16 DNA and/or p16 protein: (1) HPV type 16 negative tumors that lacked p16 expression (HPV16−/p16−); (2) HPV type 16 positive tumors that lacked p16 expression (HPV16+/p16−) and (3) HPV type 16 positive tumors that are characterized by high expression of p16 protein levels (HPV16+/p16+ oropharyngeal tumors) [37].

Despite the fact that p16 immunohistochemical expression appears to be a reliable marker for HPV infection, recent biomarker studies have shown that a small subset of HPV-negative tumors is characterized by a high p16 expression (HPV16−/P16+) [38]–[40]. The HPV16−/P16+ subgroup may represent a group of tumors with pRb loss without HPV infection, a false negativity of current HPV DNA detection methods, or a group of tumors with yet unknown molecular abnormalities in pRb/p16 cell cycle regulation.

To include this subgroup in the molecular classification of head and neck cancer, a recent comparative quantitative analysis of protein expression for 13 different biomarkers (EGFR, E-cadherin, β-catenin, MET, ERK1/2, Bcl-2, PI3Kp110, PI3Kp85, PTEN, NFκB, pAKT, STAT3, p53) was performed in p16−/HPV+, p16+/HPV+, p16+/HPV−, p16−/HPV− subgroups of SCCHN using quantitative fluorescent immunohistochemistry and automated image acquisition and analysis (AQUA). β-Catenin was again found to be significantly upregulated in p16+/HPV+ SCCHN tumors, suggesting that activation of the Wnt pathway by E6/E7 oncoproteins contributes to HPV-induced malignant transformation in host cells [41].

11.4 E6, Siah-1, and altered proteosomal degradation of β-catenin in SCCHN

In a recent study of SCCHN cell lines, transcriptionally active, nuclear β-catenin was found significantly elevated in HPV16+ versus HPV− oropharyngeal cancer cell lines, and was associated with continuous E6 and E7 oncoprotein expression, and reduced expression of Siah-1, a mediator of GSK3β-independent ubiquitination and degradation of β-catenin [42] (Fig. 11.2). Repression of HPV E6 and E7 expression by shRNA mediated silencing substantially reduced nuclear β-catenin levels. Moreover, luciferase reporter assays showed that transcriptional activation of the Tcf promoter by β-catenin was lower after the silencing of E6/E7 [42]. Independent clinicopathological studies have found that Siah-1 mRNA is decreased in tumor samples compared with normal issues [43], [44]. This work indicates that high-risk HPV E6/E7 expression markedly reduces Siah-1 protein levels in pharyngeal cells, with this reduction reversed by shRNA depletion of viral E6/E7. Notably, E6/E7 silencing in shRNA-treated cells also slightly induces steady state Siah-1 mRNA

levels (by 1.3-fold), although it is not clear whether changes involve transcriptional or posttranscriptional regulation.

P53 binds the Siah-1 promoter, inducing transcription of the gene. The Siah-1 protein forms a complex with Skp1, Eb1, Siah-interacting protein (SIP), and the APC to facilitate the degradation of β-catenin in a p53-dependent manner [45]–[47]. Under conditions of genotoxic stress, activation of p53 occurs and elevated p53 levels directly induce the expression of Siah-1 and in turn, the formation of β-catenin degradation complex [48], [49]. The expression of E6 in HPV+ oropharyngeal cancer results in the degradation of p53, limiting Siah-1 transcription, whereas shRNA depletion of E6 causes a rapid restoration of p53 protein levels [50], supporting transcriptional activation of Siah-1 gene. Smeets and colleagues [51] showed that immortalization of normal oral keratinocytes (OKCs) by HPV E6 activated the WNT pathway, with this activation associated with p53 degradation; further, a dominant negative mutant, p53(R175H), similarly activated the WNT signaling pathway.

There are numerous other examples of opposing actions of Wnt and p53 in cancer formation. For example, Wnt1 overexpressing mice bred into a p53-deficient background develop mammary adenocarcinomas at an earlier age compared to Wnt1 transgenic mice with wild type (wt) p53 function, suggesting that p53 acts upstream of Wnt to suppress its oncogenic activity [52]–[54]. Interestingly, Kim and colleagues have recently demonstrated that p53 transactivates miRNA-34 which binds the untranslated regions of Wnt effector genes, opposing the transcriptional activity of β-catenin. Therefore, loss of p53 function also increases canonical Wnt signaling by downregulating miR-34 expression [55], [56].

11.5 Degradation of pRb activates the Wnt pathway: A role for E7?

Disruption of the pRb pathway is a hallmark of many sporadic human cancers [57]. The tumor suppressor function of pRb depends on its ability to bind and repress the E2F1 transcription factor. Although the oncogenic roles of the pRb/E2F1 and Wnt/β-catenin pathways have been well studied independently, the functional interaction between the two pathways has only recently been characterized. Recent work from Costa and colleagues using a transgenic mouse specifically deficient in Rb/E2F1 in the skin ($Rb^{F/F}$; K14creER^{TM}; $E2F^{-/-}$) provides evidence that disruption of the pRb/E2F1 pathway induces activation of Wnt/β-catenin signaling. In these mice, the gene expression profile of the epidermis indicated an elevated expression of Wnt-responsive genes, accompanied by aberrantly elevated nuclear localization of β-catenin in spontaneous tumors arising in the skin after acute loss of pRb and E2F1 [58]. These data clearly suggest that E7-driven degradation of pRb in HPV+ SCCHN may also increase β-catenin signaling, although the pathway as yet remains unknown.

11.6 In vivo models of HPV-induced stabilization of β-catenin

The crucial role of viral E6 expression in activation of the WNT signaling pathway in HPV type 16 positive epithelia has also been confirmed in a transgenic mouse model that expresses the E6 oncoprotein in the basal layer of stratified epithelia under the control of human keratin 14 promoter (K14E6 mice) [59]. Mice from a transgenic E6 strain were shown to display skin hyperplasia and skin cancer at advanced ages, associated with nuclear accumulation of β-catenin and the transcriptional activation of β-catenin-responsive genes in skin epidermis [59]. In contrast, mice from a transgenic strain expressing a truncated E6 oncoprotein that lacks the PDZ binding domain (K14E6ΔPDZ mice) induces neither nuclear β-catenin nor the transcription of β-catenin-responsive genes. The C-terminal PDZ domain of E6 found in all high-risk HPV variants confers interaction with a large number of PDZ-containing cellular proteins, including cell polarity and cell survival regulators such as NHERF1, DLG1, Scribble, TIP-2, and others [60]. How these interactions contribute to viral control of WNT signaling is currently not clear.

The synergy between the WNT signaling pathway and HPV was also shown in an additional in vivo study using mice that use the Keratin 14 (K14) promoter to express either the HPV16 E7 oncoprotein (K14E7), a constitutively active amino-terminal truncated β-catenin molecule that lacks the four phosphorylation sites required for its degradation (K14-ΔN87βcat), or both (K14-E7ΔN87βcat) [61]. In this study, the K14-ΔN87βcat transgenic strain developed benign skin tumors but did not exhibit histopathologic characteristics of cervical cancer while the K14-E7 strain displayed cervical pathologies after 6 months of estrogen treatment. The authors analyzed the incidence of invasive cervical cancer in K14-ΔN87βcat, K14-E7, and K14-E7ΔN87βcat mice at 7 months of age, finding invasive cervical cancer developed in 11, 50, and 94 % of the transgenics, respectively. These data suggest that the activation of WNT pathway accelerates HPV16-E7-mediated cervical carcinogenesis.

11.7 Conclusions

HPV-associated SCCHN represents a distinct entity from tobacco- and alcohol-related SCCHN, with a very different molecular profile. HPV has evolved to coexist within epithelial cells and its life cycle is tightly linked to proliferation and differentiation of the basal epithelial cells. E6 and E7 viral oncoproteins modulate cellular signaling pathways that regulate the proliferation capacity and the differentiation state of keratinocytes. The Wnt signaling pathway is of central importance in regulating epithelium differentiation, and HPV uses components of this pathway to alter cell gene expression by manipulation of downstream pathway responses. Recent experimental studies on HPV-associated SCCHN provide evidence that HPV-expressed viral proteins promotes β-catenin stabilization, inhibiting its proteosomal degradation. These activities require the virally encoded E6/E7 proteins, which induce the

degradation of p53 and pRb, negative regulators of Wnt pathway. Further investigation of the interconnection between the Wnt pathway and E6/E7 is needed. The understanding of viral manipulation of the Wnt pathway in HPV infected head and neck cells may provide opportunities to create new molecular markers for HPV diagnostics and new therapeutic approaches.

References

1. Wilson VG, et al. Papillomavirus E1 proteins: form, function, and features. Virus Genes. 2002;24(3):275–90.
2. De Geest K, et al. Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines. Gynecol Oncol. 1993;49(3):303–10.
3. Stanley MA, et al. Properties of a non-tumorigenic human cervical keratinocyte cell line. Int J Cancer. 1989;43(4):672–6.
4. Zhang P, et al. Induction of E6/E7 expression in cottontail rabbit papillomavirus latency following UV activation. Virology. 1999;263(2):388–94.
5. Gravitt PE. The known unknowns of HPV natural history. J Clin Invest. 2011;121(12):4593–9.
6. Tang AL, et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. Head Neck. 2012;34(10):1480–91.
7. Kimelman D, Xu W. Beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene. 2006;25(57):7482–91.
8. Skapek SX, et al. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science. 1995;267(5200):1022–4.
9. Zeilstra J, et al. Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. Cancer Res. 2008;68(10):3655–61.
10. Shtutman M, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A. 1999;96(10):5522–7.
11. Mann B, et al. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc Natl Acad Sci U S A. 1999;96(4):1603–8.
12. Wilkins JA, Sansom OJ. C-Myc is a critical mediator of the phenotypes of Apc loss in the intestine. Cancer Res. 2008;68(13):4963–6.
13. Lindsley RC, et al. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development. 2006;133(19):3787–96.
14. Zhu AJ, Watt FM. Beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development. 1999;126(10):2285–98.
15. Reya T, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003;423(6938):409–14.
16. Silva-Vargas V, et al. Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells. Dev Cell. 2005;9(1):121–31.
17. Alonso L, Fuchs E. Stem cells of the skin epithelium. Proc Natl Acad Sci U S A. 2003;100(Suppl 1):11830–5.
18. Ito M, et al. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature. 2007;447(7142):316–20.
19. Narhi K, et al. Sustained epithelial beta-catenin activity induces precocious hair development but disrupts hair follicle down-growth and hair shaft formation. Development. 2008;135(6):1019–28.
20. Zhang Y, et al. Activation of beta-catenin signaling programs embryonic epidermis to hair follicle fate. Development. 2008;135(12):2161–72.
21. Hayward SD, Liu J, Fujimuro M. Notch and Wnt signaling: mimicry and manipulation by gamma herpesviruses. Sci STKE. 2006;2006(335):re4.

22. Jang KL, et al. Up-regulation of beta-catenin by a viral oncogene correlates with inhibition of the seven in absentia homolog 1 in B lymphoma cells. Proc Natl Acad Sci U S A. 2005;102(51):18431–6.
23. Angelova M, et al. Human cytomegalovirus infection dysregulates the canonical Wnt/beta-catenin signaling pathway. PLoS Pathog. 2012;8(10):e1002959.
24. Castellsague X, et al. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. J Natl Cancer Inst. 2006;98(5):303–15.
25. Shinohara A, et al. Cytoplasmic/nuclear expression without mutation of exon 3 of the beta-catenin gene is frequent in the development of the neoplasm of the uterine cervix. Gynecol Oncol. 2001;82(3):450–5.
26. Pereira-Suarez AL, et al. Frequent alterations of the beta-catenin protein in cancer of the uterine cervix. Tumour Biol. 2002;23(1):45–53.
27. Chung MT, et al. SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. Gynecol Oncol. 2009;112(3):646–53.
28. Ueda M, et al. Mutations of the beta- and gamma-catenin genes are uncommon in human lung, breast, kidney, cervical and ovarian carcinomas. Br J Cancer. 2001;85(1):64–8.
29. Su TH, et al. Mutation analysis of CTNNB1 (beta-catenin) and AXIN1, the components of Wnt pathway, in cervical carcinomas. Oncol Rep. 2003;10(5):1195–200.
30. Imura J, et al. Beta-catenin expression as a prognostic indicator in cervical adenocarcinoma. Int J Mol Med. 2001;8(4):353–8.
31. Shim C, et al. Profiling of differentially expressed genes in human primary cervical cancer by complementary DNA expression array. Clin Cancer Res. 1998;4(12):3045–50.
32. Perez-Plasencia C, Duenas-Gonzalez A, and B Alatorre-Tavera. Second hit in cervical carcinogenesis process: involvement of wnt/beta catenin pathway. Int Arch Med. 2008;1(1):10.
33. van der Meide WF, et al. Promoter methylation analysis of WNT/beta-catenin signaling pathway regulators to detect adenocarcinoma or its precursor lesion of the cervix. Gynecol Oncol. 2011;123(1):116–22.
34. Klussmann JP, et al. Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. Am J Pathol. 2003;162(3):747–53.
35. Hafkamp HC, et al. A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5–8. Int J Cancer. 2003;107(3):394–400.
36. Worsham MJ, et al. Fine-mapping loss of gene architecture at the CDKN2B (p15INK4b), CDKN2A (p14ARF, p16INK4a), and MTAP genes in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2006;132(4):409–15.
37. Weinberger PM, et al. Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favorable prognosis. J Clin Oncol. 2006;24(5):736–47.
38. Singhi AD, Westra WH. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer. 2010;116(9):2166–73.
39. Ang KK, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363(1):24–35.
40. Rischin D, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol. 2010;28(27):4142–8.
41. Rampias T, et al. Molecular profile of head and neck squamous cell carcinomas bearing p16 high phenotype. Ann Oncol. 2013;24(8):2121–31.
42. Rampias T, et al. Activation of Wnt signaling pathway by human papillomavirus E6 and E7 oncogenes in HPV16-positive oropharyngeal squamous carcinoma cells. Mol Cancer Res. 2010;8(3):433–43.
43. Bruzzoni-Giovanelli H, et al. Distinct expression patterns of the E3 ligase SIAH-1 and its partner Kid/KIF22 in normal tissues and in the breast tumoral processes. J Exp Clin Cancer Res. 2010;29:10.

44. Brauckhoff A, et al. [Reduced expression of the E3-ubiquitin ligase seven in absentia homologue (SIAH)-1 in human hepatocellular carcinoma]. Verh Dtsch Ges Pathol. 2007;91:269–77.
45. Liu J, et al. Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol Cell. 2001;7(5):927–36.
46. Matsuzawa SI, Reed JC. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. Mol Cell. 2001;7(5):915–26.
47. Maeda A, et al. The characterization of the human Siah-1 promoter(1). FEBS Lett. 2002;512(1–3):223–6.
48. Dimitrova YN, et al. Direct ubiquitination of beta-catenin by Siah-1 and regulation by the exchange factor TBL1. J Biol Chem. 2010;285(18):13507–16.
49. Wang D, et al. An upregulation of SIAH1 after spinal cord injury in adult rats. J Mol Neurosci. 2011;45(2):134–44.
50. Rampias T, et al. E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells. J Natl Cancer Inst. 2009;101(6):412–23.
51. Smeets SJ, et al. Immortalization of oral keratinocytes by functional inactivation of the p53 and pRb pathways. Int J Cancer. 2011;128(7):1596–605.
52. Donehower LA, et al. Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev. 1995;9(7):882–95.
53. Jones JM, et al. Absence of p53 in a mouse mammary tumor model promotes tumor cell proliferation without affecting apoptosis. Cell Growth Differ. 1997;8(8):829–38.
54. Gunther EJ, et al. Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev. 2003;17(4):488–501.
55. Kim NH, et al. p53 regulates nuclear GSK-3 levels through miR-34-mediated Axin2 suppression in colorectal cancer cells. Cell Cycle. 2013;12(10):1578–87.
56. Kim NH, et al. p53 and microRNA-34 are suppressors of canonical Wnt signaling. Sci Signal. 2011;4(197):ra71.
57. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell. 1995;81(3):323–30.
58. Costa C, et al. E2F1 loss induces spontaneous tumour development in Rb-deficient epidermis. Oncogene. 2013;32(24):2937–51.
59. Bonilla-Delgado J, et al. The E6 oncoprotein from HPV16 enhances the canonical Wnt/beta-catenin pathway in skin epidermis in vivo. Mol Cancer Res. 2012;10(2):250–8.
60. Pim D, et al. Human papillomaviruses and the specificity of PDZ domain targeting. FEBS J. 2012;279(19):3530–7.
61. Bulut G, et al. Beta-catenin accelerates human papilloma virus type-16 mediated cervical carcinogenesis in transgenic mice. PLoS One. 2011;6(11):e27243.

Chapter 12
p53 in Head and Neck Cancer

Jong-Lyel Roh and Wayne M. Koch

Abstract Alteration of the *TP53* tumor suppressor gene is a central event in the development of human malignancy. The majority of squamous cell carcinomas of the head and neck (HNC) have undergone this molecular disruption either by point mutation of *TP53* or through disruption of p53 protein function as a consequence of oncogenic human papillomavirus (HPV) infection. p53 interactions with partner proteins control key cellular pathways that affect apoptosis, the cell cycle, and proliferation, particularly in response to stress. Early research demonstrated mutation in the coding region of *TP53* in more than half of all HNC tumors. However, the anticipated value of *TP53* alteration for clinical management of HNC has not materialized. This is due, in part, to the complexity of *TP53* alterations, which occur at numerous loci within the gene and produce highly variable results with regard to protein function, as well as to the highly integrated position of p53 within densely connected cellular pathways that induce pleiotropic effects. Furthermore, as a tumor suppressor, p53 is not immediately accessible as a therapeutic target, since the restoration of protein activity is more difficult to achieve than the blockage of activity of a gain-of-function event. However, interest in *TP53* alteration in HNC has undergone a minor renaissance with the observation that some mutations which disrupt the DNA-binding function of p53 are associated with more aggressive cancer phenotype than are mutations that are nondisruptive. Identification and targeting of this class of *TP53* mutation may, hence, have clinical importance in the disease management of HNC. We summarize current relevant issues in *TP53* biology.

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12.1 Introduction

The p53 protein, encoded by the *TP53* gene, has a pivotal role in tumor suppression and is a mainstay of intrinsic anticancer defense systems. Its importance is reflected in the popular name "guardian of the genome" [1]. This 53 kDa protein was first identified through studies of Simian virus 40 large T antigen in 1979 that identified its induction [2]–[6], then later recognized as a cellular oncoprotein. p53 was initially thought to be a positive effector of malignant transformation, as many tumors and transformed cell lines produce high levels of p53 relative to normal tissues [7], [8]. The misclassification of p53 as an oncogene was uncovered by the subsequent observation that the loss of this gene promotes cancer [9]. The extensive analysis of DNA from human cancers, coupled with in vitro functional assays, demonstrated that the wild-type *TP53* alleles were frequently mutated in different cancer types [10], [11]. Further, investigation of *Trp53* knockout mice [12]–[14] and humans afflicted with Li–Fraumeni syndrome [15], which is linked to *TP53* germline mutations [16]–[18], subsequently established p53 as a bona fide tumor suppressor.

Besides direct mutation of the *TP53* gene, p53 levels can be grossly reduced based on posttranslational regulation arising from altered expression of p53-binding partners. Cellular p53 levels are controlled by the ubiquitin-proteasome pathway, which in the mouse are regulated through the binding of the murine double minute 2 protein (MDM2; the human protein is called HDM2). MDM2 forms a negative-feedback loop as a direct transcriptional target of p53, thereby promoting the degradation of p53 and suppressing its cellular activity [19]–[22] (Fig. 12.1). In contrast, p53 is rapidly activated in cells exposed to various stress conditions through the abrogation of the inhibitory effects of MDM2 binding and via the activation of alternative reading frame (ARF), a tumor suppressor protein that also binds to MDM2 and thereby augments the cellular levels and function of p53 [23]. MDMX (also known as MDM4), which is related to MDM2, binds to the amino-terminal region of p53 and inhibits its activity by augmenting the E3 ligase activity of MDM2 [24]–[25]. The excessive expression of MDM2 or MDM4 thus contributes to human cancer by disrupting the intricate interplay of MDM2 and p53 [26].

Head and neck cancer (HNC) is the eighth most common cancer worldwide, with more than half a million new cases diagnosed each year [27]. The overall incidence of HNC in the USA is declining despite a rising incidence of oropharyngeal cancer associated with oncogenic human papillomavirus (HPV) [28], [29]. Tobacco and alcohol consumption increase the risk of developing traditional HNC, which is largely attributable to the genotoxic effects of the carcinogens in these substances [30]. Over 50 % of HNCs demonstrate chromosomal loss at 17p, the site of the *TP53* gene, and harbor inactivating *TP53* gene mutations [31], [32]. Conversely, the posttranslational

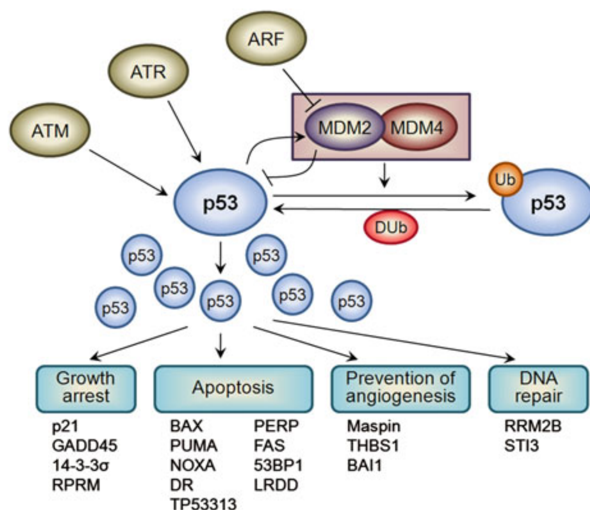

Fig. 12.1 The *p53-MDM2* negative-feedback loop is at the center of the p53 pathway. In non-stressed conditions, the p53 levels and activities remain low through its ubiquitination (Ub) by MDM2 and *MDM4,* both of which bind p53. Under stressed conditions, upstream signaling to p53 increases its levels and activities and the function of the MDM2-MDM4 complex is blocked. p53 activation is closely related to various stress signals including DNA damage, oxidative damage, oncogene activation, hypoxia, nitric oxide, ribonucleotide depletion, telomere erosion, etc. p53 then transactivates and represses a number of target genes and protein–protein interactions, such as those involved in apoptosis and that induce various phenotypic changes

inactivation of wild-type p53 is induced in tumors infected with oncogenic strains of HPV (including HPV16 and HPV18), with the HPV- encoded protein, E6, acting analogously to MDM2 , as a ubiquitin ligase to promote p53 degradation[33]. *TP53* mutations are observed mainly in HPV-16-negative HNCs that are generally less responsive to chemoradiation and show poor survival when compared with HPV-16-positive head and neck squamous cell carcinoma (HNSCCs). The contribution of p53 status to the improved prognosis of HPV-related HNC may be explained in several ways. Inactivation of p53 by E6 HPV protein may be partial, allowing for the retention of some normal function. Furthermore, cells in which p53 is inactivated by HPV will not experience any gain of function of p53 that may occur with certain mutations. In normal cells, the p53 tumor suppressive function is activated by a variety of cellular damage triggers, and exerted via the induction of cell-cycle arrest, senescence, and apoptosis in response to oncogene activation, DNA damage, and other stress signals. Hundreds of genes transactivated by p53 are intimately involved in apoptosis or cell-cycle control, including the proapoptotic protein Bcl2-associated X protein (BAX) and cyclin-dependent kinase inhibitor p21 [34], [35]. Activated p53 also interacts in the cytoplasm with the Bcl2 family members, directly contributing to cellular apoptosis by increasing mitochondrial membrane permeabilization and cytochrome c release [36]. Hence, tumor cells lacking functional p53 are resistant to apoptosis.

Accordingly, the *TP53* mutation status in some tumors has been demonstrated to be a reliable predictor of the chemotherapy response and patient prognosis [32], [37]–[39]. DNA-damaging agents routinely used for cancer therapy engage the pivotal role of p53 in their mode of action to kill the cancer cell. A recent study indicates that head and neck tumor cell lines with *TP53* mutations that disrupt DNA binding are significantly more radioresistant than cell lines with wild-type *TP53* [40]. However, the p53 pathway is often affected in tumors without direct *TP53* mutations, clouding the assessment of its contribution to clinical tumor behavior.

Over the last few decades, p53 has been a valuable target for human cancer therapy development. *TP53* gene transfer with an adenovirus vector was approved in 2004 for the treatment of HNC in China [41], and the outcomes of clinical trials with Advexin or ONYX-015 are eagerly awaited [42], [43]. Restoring p53 activity has also been attempted by the development of low-molecular-mass compounds that interact with mutant p53 or the p53-MDM2 pathway. Clinical trials of the efficacy of such compounds are underway, including PRIMA-1, which interacts with and stabilizes p53 mutants, and nutlins, which interact with the p53 binding pockets of the MDM2 molecule and dislodge p53 from MDM2 [44], [45]. Other compounds leading to the restoration of p53 function in cancer cells are in development.

In this review, we describe the functional elements of p53 structure and then address the pattern of mutations affecting p53 structure and activity. We then discuss the p53 family members, p63, p73, and p53 isoforms, and growing evidence for their important roles in cancer. We outline important p53 protein interactions that are essential for its tumor suppressive activity. In the context of these data, we then discuss issues relevant to the use of p53 as a biomarker for cancer prognosis and treatment response, and development of p53 as a therapeutic target. In each case, we emphasize the importance of findings in the context of HPV-negative versus HPV-positive HNC.

12.2 p53 Structure

Within normal human cells, the p53 protein exists as a tetramer of four identical chains of 393 residues consisting of three main functional domains: an amino (N)-terminal region harboring the transactivation domain (TAD) and a proline-rich region (residues 1–61 and 64–92, respectively); a sequence-specific DNA-binding domain (DBD) (residues 110–286); and a carboxyl (C)-terminal region including a nuclear localization signal and an oligomerization domain (residues 326–355 and 363–393, respectively) [46] (Fig. 12.2).

The acidic TAD binds various transcription factors, the transcriptional coactivators p300 and CRP, and the negative regulators MDM2 and MDM4. The MDM2-binding region of the TAD overlaps with the binding site for the transcriptional coactivator p300. Hence, p300 and MDM2 compete for the same binding site, and p300 thus protects p53 from MDM2-mediated degradation. Conversely, MDM2 prevents p300 or other transcriptional machinery from binding. The transactivating

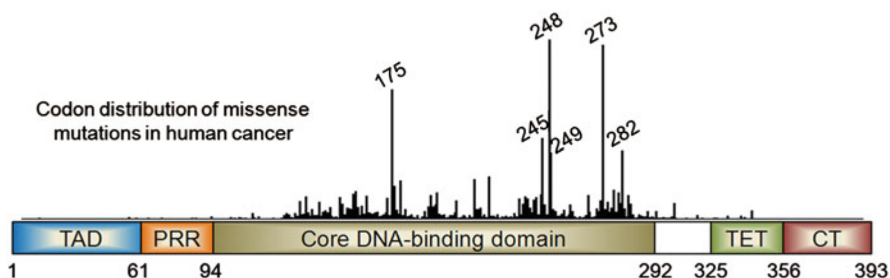

Fig. 12.2 Schematic diagram of full-length p53 consisting of an N-terminal transactivation domain (*TAD*), a proline-rich region (*PRR*), the central DNA-binding domain, the tetramerization domain (*TET*), and the extreme C-terminus (*CT*). The codon distribution of single base substitutions in human cancer ($n = 22{,}356$) were derived from the IARC p53 mutation database. (www.iarc.fr/p53, the latest version, R15, was released in 2010)

signal produced by p53 depends on the concentrations of proteins competing for its N-terminus. TAD mutations weaken the binding of p53 to p300 domains and impair protein–protein interactions [47]. The proline-rich region contains five PXXP motifs that mediate numerous protein–protein interactions with Src homology 2 domains, and are responsible for p53 interactions with stabilizing proteins. The function of this region is poorly understood, but it is known to be indispensable for tumor suppression [48].

The central core DNA-binding domain of p53 consists of an immunoglobulin-like β-sandwich including a surface with two structural motifs with minor and major grooves for binding target DNA. The DNA major groove docks with the loop-sheet-helix motif including loop L1, β-strands, and the C-terminal helix. The other half of the DNA-binding groove is formed by two large loops, L2 and L3, which are stabilized by a zinc ion with coordination by His-179, Cys-176, Cys-238, and Cys-242. The loss of zinc results in a significant decrease in thermodynamic stability causing a loss of DNA-binding specificity [49]. p53 regulation of gene transcription can be either positive or negative, depending on the nature and context of the binding element present. Two core domains bind to symmetrical half-site dimers in DNA-response elements with different space lengths between two half sites [50]. The key residues in the DNA-binding domain that make direct contact with a DNA half-site are Lys-120, Ser-241, Arg-248, Arg-273, Ala-276, Cys-277, and Arg-280. The acetylation of Lys-120, located on loop L1, plays a role in the induction of apoptosis [51]. p53-regulated gene expression is further modulated by interaction with the ASPP family which regulates the apoptotic response and chromatin state [52].

The tetramerization domain in the C-terminal region of p53 consists of a short β-strand and an α-helix linked by a sharp turn (Gly-334). A primary dimer, formed by two monomers, is stabilized via an antiparallel β-sheet and helix packing with three residues (Leu-330, Ile-332, Phe-341). Two dimers form a tetramer stabilized by hydrophobic interactions and the truncation of two key residues (Leu-344 and Leu-348) to shift the oligomerization to form stable dimers [53]. A dimeric mutation in p53 may form a different tetramer to alter the orientation of the β-strand and the α-helix, thus preventing co-oligomerization with wild-type proteins [54].

The extreme C-terminal end of p53 binds to other proteins or nonspecifically to DNA. The p53 residues 367–388 form a complex with the S100B protein and the bromodomain of CBP, while residues 376–386 bind to phospho-CDK1/cyclin A [55]. The PGGS motif, residues 359–362, binds to HAUSP/USP7, which is recognized by MDM2-derived peptides [56]. The C-terminal region of p53 is subject to extensive posttranslational modifications to regulate its function and cellular protein levels. Acetylation of this region enhances DNA-binding activity and recruits coactivators/histone acetyltransferases for the transcriptional activation of target genes [57]. Truncation of the C-terminal region reduces DNA binding and lowers the transactivation of p53 target genes [58]. Modifications of the p53 C-terminal region may contribute to the fine-tuning of the p53 stress response and reflect the complexity of the p53 pathway, including the regulation of nuclear p53 levels, changes in DNA binding, and coactivator recruitment.

12.3 Spectrum of Mutations in HNC

The *TP53* database of the International Agency for Research on Cancer (IARC), version R11, reports more than 17,000 cases of p53 missense mutations, mostly clustered in the DNA-binding domain [59]. The phenotypes associated with common p53 cancer mutations are diverse, ranging from complete loss of its function to promoter-dependent activity at subphysiological temperature [60]. Moreover, there is increasing evidence that particular types of p53 mutations correlate with the therapeutic response and cancer prognosis [32], [37], [40].

Hotspot p53 mutations found in tumors are classified as either contact or structural mutations. Contact mutations involve the alteration of DNA-contact residues (R248Q, R248W, R273H, and R273C), whereas, structural mutations affect residues required for the overall architecture of the DNA-binding surface of p53 (R175H, G245S, R249S, and R282W) [61]. Many oncogenic mutations in the β-sandwich region (V143A, V157F, Y220C, F270C, etc.) reduce the thermodynamic stability of p53 and its binding to DNA response elements. Mutations affecting zinc ligands (C242S) or the zinc-binding site (R175H) abrogate DNA binding by p53 in its folded state. The removal of Arg-273, an essential DNA-contact residue in p53, does not perturb the overall architecture of the DNA-binding surface but reduces the binding specificity to gadd45 DNA by about 1,000-fold *in vitro* [62].

The L3 loop of p53, which is the DNA minor-groove-binding region, is the site of structural hotspot mutations (G245S and R249S). Mutational changes at G245 affect residues at the dimerization interface in the p53-DNA complex and weaken DNA binding by reducing binding cooperativity [63]. R249 plays a crucial role in stabilizing L3 loop conformation, and its mutation leads to highly flexible and substantial conformational changes in the L3 loop, which result in substantial impairment of DNA binding by p53 and stability loss [64]. Mutational changes in R282 perturb the stability of the loop-sheet-helix motif to the β-strand S2 and loop L1 without changes in the overall architecture of the remaining DNA-binding region of p53 [63].

Mutations in the β-sandwich region of p53 (V143A and F270L) are relatively uncommon but create internal cavities in the hydrophobic core of this region without collapse of the surrounding structure, which results in the loss of hydrophobic interactions [63]. Y220C is the most frequent cancer mutation in p53 outside the DNA-binding surface and has a similar destabilizing effect. Both the V143A and Y220C substitutions elicit temperature-sensitive phenotypes that are inactive at body temperature but initiate promoter-dependent transactivation activity at subphysiological temperatures [65]. Hence, many mutations in the β-sandwich region of p53 primarily result in a lower intrinsic stability and thermodynamic stability of p53 that cause more benign phenotypes [63].

The prevalence of p53 mutations in HNSCC varies from 30–70 % according to different levels of exposure to risk factors in study populations [32], [66]–[71]. The frequency of p53 mutations is the greatest among patients who smoke and drink alcohol [66]. In contrast, *TP53* mutations have been found to be inversely associated with the HPV status of tumors [72], [73]. HPV-positive tumors may also contain *TP53* mutation in a minority of cases (25 %), but these mutations tend to be "nondisruptive," not altering the predicted DNA-binding capability of the resultant p53 protein [73]. Numerous tobacco carcinogens and environmental chemicals increase the incidence of somatic mutations in the p53 gene, particularly in smokers with CYP1B1, GSTP1, or XRCC1 genotypes [60], [70], [74]. The pattern and codon distribution of p53 mutations encountered in HNSCC is shown in Fig. 12.3. The most prevalent point mutations are G:C > A:T transitions and G:C > T:A transversions. Frameshift mutations (deletions and insertions) occur in approximately 16 % of HNSCCs, compared with 9 % in most other cancers [59] but equivalent to the prevalence in esophageal squamous cell carcinoma. These types of mutations are also more frequently seen in subgroups of patients who consume alcohol and tobacco [75]. In an analysis of 1,576 single base substitutions from the IARC p53 mutation database, the most frequent *TP53* mutations in HNSCCs share six hot spot p53 residues (175, 245, 248, 249, 273, and 282) found in other cancers [76] and additional mutations are found at residues 179 and 220 [59].

12.4 Loss and Gain of Function p53 Mutants

The majority of *TP53* cancer mutations abrogate the DNA-binding activity of wild-type p53 response elements as a loss-of-function (LOF) effect [60]. These mutant p53 proteins acquire a dominant-negative (DN) activity over the remaining wild-type species and also gain new oncogenic properties ("gain of function," GOF) that are independent of wild-type p53. Hence, these p53 mutations not only result in the abrogation of wild-type p53 function, but the mutant p53 proteins also gain oncogenic functions [9], [77]–[79](Fig. 12.4). There is growing evidence that GOF *TP53* mutations in human cancer are associated with drug resistance and poor clinical outcomes [32], [37]–[38], [80].

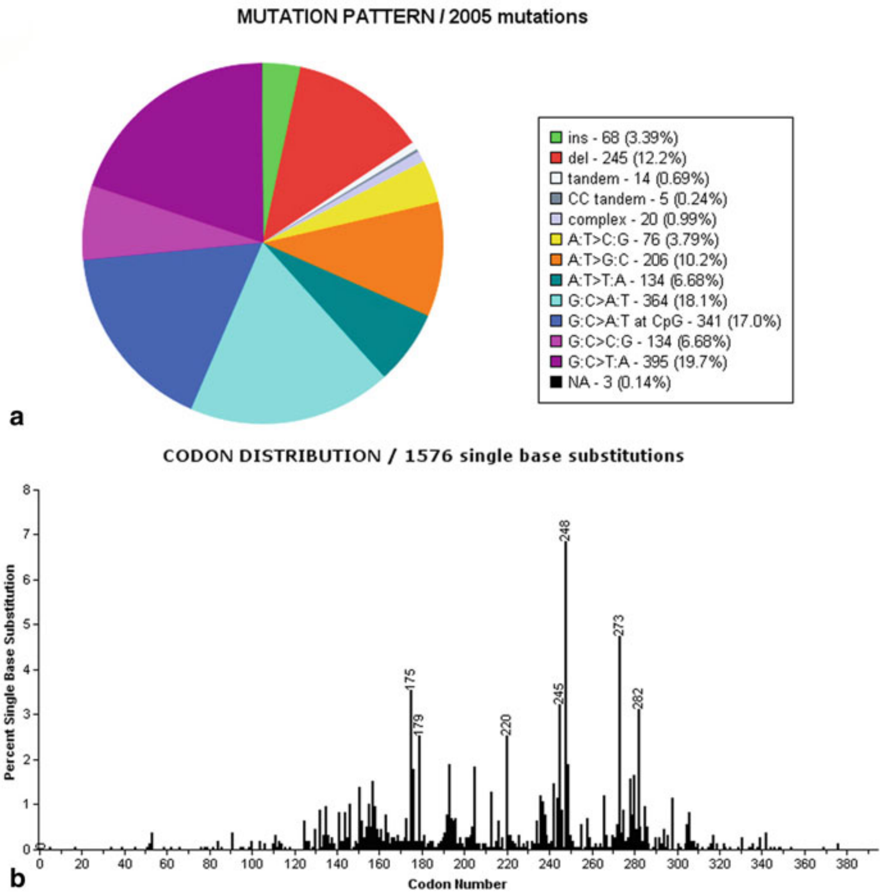

Fig. 12.3 The pattern (**a**) and codon distribution (**b**) of p53 mutations encountered in HNSCC. Data were derived from the IARC p53 mutation database. (www.iarc.fr/p53, the latest version, R15, was released in 2010)

The high proportion of missense mutations at *TP53* hotspot residues favors the gain-of-function hypothesis and the inactivation of DNA-binding capability is more likely to result from missense mutations than truncating mutations. The mutagenic effects of smoking (benzo-[a]-pyrene) or aflatoxin B1 food contamination may affect point mutations at different regions of the *TP53* locus: V157F and R158L substitutions are frequent in lung cancer while the R249S substitution is extremely common in liver cancer [78]. In HNC, the rate of *TP53* mutation among cancers of smoking and drinking patients is significantly greater than among abstainers (58 vs. 17 %), and the pattern of mutation among nonsmokers suggests an endogenous mutation mechanism, appearing largely at CpG islands [66]. The unique pattern and mode of function of *TP53* mutations is shaped by intrinsic factors and environmental carcinogens, as well as by a complexity that is yet to be fully understood in tumors.

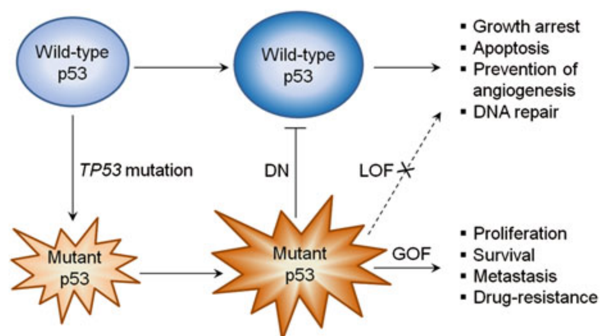

Fig. 12.4 The phenotypic effects of *TP53* mutations observed in human tumors. First, most *p53* mutations abrogate the capacity of p53 to bind its consensus DNA sequence and thus impede its transcriptional activity (loss of function, *LOF*). Second, p53 point mutations produce a dominant-negative (*DN*) effect over the remaining wild-type allele through the oligomerization of the mutant and wild-type proteins. Finally, p53 mutants gain new functions (gain of function, *GOF*) independently of wild-type p53 that promote tumor growth, metastasis, drug-resistance, and survival

Wild-type p53 is commonly maintained at low levels in unstressed normal tissues with a short half-life of 9 min [81], which results from ubiquitin-mediated degradation of p53 via the E3 ubiquitin ligase and the wild-type p53 target gene MDM2 [21], [82]. In contrast, mutant p53 proteins show an extremely prolonged half-life and often accumulate at extremely high levels in tumors due to the disruption of normal negative feedback mechanisms by mutations that abrogate the ability of p53 to transactivate MDM2 [83]–[86]. The stabilization of mutant p53 proteins is determined by a chaperone, such as HSP70 and HSP90, that interacts with mutant p53 to accumulate p53 and inhibit MDM2.

Hetero-oligomerization with mutant $p53^{R172H}$ inhibits the ability of two p53-related proteins, p63 and p73 (discussed below), to induce cell-cycle arrest and suppress focus formation. p53 mutants that retain the ability to bind p73 protect cancer cells from chemotherapeutic agents and thus produce a less favorable response to chemoradiotherapy in HNC patients [87]. The binding capacity of different p53 mutants for p73 is influenced by the site of the mutation and single nucleotide polymorphisms at codon 72 [88]. For example, the $p53^{V143A}$ and $p53^{R175H}$ mutants with a 72R polymorphism bind to p73 more efficiently than those carrying 72P [88]. Squamous cell carcinomas bearing p53/72R mutants show a poorer prognosis than those harboring 72P. Interference with such mutant p53–p73 interactions restores the activity of p73 resensitizing cancer cells to chemotherapy and thereby hindering cancer cell growth [89], [90].

Although hotspot mutations in the DNA-binding domain of p53 abrogate its activation of target genes, they can also modulate gene transcription and interfere with pivotal signaling pathways by which p53 mutants exert their oncogenic functions [78], [91]. A functional transcription-activation domain of p53 is required for the activation of *MDR1* by mutant p53 to protect cancer cells from apoptosis induced by chemotherapeutic agents [92]. Transcription factor NF-Y interacts with the $p53^{R175H}$

and p53^{R273H} mutants as well as wild-type p53, which explains the growth-promoting properties and insensitivity of p53 mutants to DNA damage [37], [80]. In addition, mutant p53 inhibits the ability of wild-type p53 to repress factors involved in inflammation, such as cytokines, chemokines, and extracellular matrix modulators. Furthermore, it induces the transcription of inflammatory genes through the activation of the nuclear factor (NF)-κB pathway, enhancing the NF-κB response to tumor necrosis factor α (TNFα) and activating *NEKB2* [93]. The p53^{R248W} and p53^{R273H} hotspot mutants bind MRE11, in the ataxia telangiectasia mutated (ATM)-dependent DNA-damage response pathway, and consequently inhibit the cellular response to DNA double-stranded breaks. This phenomenon is not observed in *Trp53*-null mice [94]. These interactions of mutant p53 with transcriptional genes, binding proteins, and the DNA-damage response network may account for the emerging molecular signatures providing mechanisms underlying the gains of function associated with *TP53* mutations in human malignancies.

12.5 p53 Family Proteins p63 and p73, and Isoforms of p53

The p53 family members p63 and p73 have DNA-binding domains that are closely related to p53, bind to similar DNA sequences, and can induce the transcription of different genes [95], [96]. These p53 family members have tissue-specific essential roles in the normal development of the nervous system, in the skin and female reproductive organs, and as tumor suppressors under some circumstances [95]. p63 or p73-null mice die within a few months of birth, whereas, p53-null mice undergo largely normal embryonic development but die of cancer at a young age. Loss of the p63 gene affects the pool of proliferating stem cells during the development of epithelium, with a subsequent lack of limbs and disruptions to a wide range of epithelial structures of the skin, breast, urothelium, etc. [97]. The deletion of *Trp73* in mice also results in nervous system abnormalities, reproductive and behavioral defects, and immunological defects associated with chronic inflammation [98].

The architecture of p63 and p73 is similar to that of p53 [95]. However, the oligomerization domain of p53 has sequence-nonspecific binding ability while both p63 and p73 possess a sterile alpha motif (SAM) domain with protein–protein interaction properties. p63 and p73 share a high degree of sequence identity with each other, and within the DBD, a 65 % amino-acid identity with p53. The role of p63 and p73 in tumor suppression is less obvious than that of p53, and mutations of p63 and p73 have not been associated with higher tumor incidence [99]. However, there is growing evidence that these p53-related proteins influence tumor suppression. Full length, transactivating-competent isoforms of p63 and p73, termed TAp63 and Tap73, are thought to contribute to cell-cycle arrest, senescence, apoptosis, and DNA repair in response to DNA damage stimuli, independent of p53 [100], [101]. The p73-E2F1 pathway is involved in chemotherapy-induced apoptosis and tumor chemosensitivity. Aging p63$^{+/-}$ or p73$^{+/-}$ heterozygote mice spontaneously succumb to spontaneously forming tumors, with a median survival time a few months longer than that of p53$^{+/-}$ mice [99].

All three of the p53 family proteins exist in many differently spliced isoforms. p53 itself does not exist as a single protein but produces nine different isoforms containing combinations of N-terminal and C-terminal truncated species through alternative splicing and transcriptional initiation [102]. For each protein, an isotype with a truncated N-terminus, the ΔN protein (Δ133p53, ΔNp63, and ΔNp73) and further ΔN isoforms (Δ40p53, Δex2p73, Δex2/3p73, and ΔN′p73) is generated by alternative splicing and initiation of translation. The transactivation-compromised ΔN isoforms are functionally distinguishable from the full-length isoforms (FLp53, Tap63, and Tap73) by the loss of antiapoptotic and dominant-negative properties. Different isoforms (α, β, γ, δ, ε, ζ, and η) caused by the alternative splicing of C-terminal exons are also known, but their biological functions and transcriptional activities are still incompletely understood. Truncated N-terminal p53 variants act as dominant-negative repressors of p53-regulated genes [103].

TAp63 has been identified as a potent transforming growth factor (TGFβ)-dependent suppressor of invasiveness and metastasis of epithelial tumors, controlling the expression of a crucial set of metastasis-inhibitor genes [104]. Opposing this activity, the ΔN isoforms of p53 family proteins are overexpressed in some tumors, which suggests a common mechanism for inactivation of the TA isoforms might substitute for mutational inactivation [105]. For instance, the ΔNp63 isotype is responsible for the maintenance of the proliferative potential of basal keratinocytes, and its expression is lost as keratinocytes differentiate by arresting the cell cycle [106]. Altered expression of TAp63 or its ΔN isoform is found in squamous cell carcinoma of the head and neck, esophagus, and lung. p63 is overexpressed in 80–100 % of HNSCC in the nonkeratinizing area of the tumors [107], [108]. The overexpression of ΔNp63 in precancerous lesions, such as leukoplakia, lichen planus, actinic cheilitis, or erythroplakia, is correlated with an increased risk of developing oral cancer, and oral cancers that develop from ΔNp63-negative precancerous lesions showed more favorable outcomes than ΔNp63-positive oral cancer [109]. Further, oncogenic mutant p53 can also interfere with the functions of p63 and p73, subsequently promoting transformation, metastasis, and chemoresistance. Recently, ΔNp63α, a dominant inhibitory isoform of p63, is regulated by IKKβ, thereby rendering HNC cells susceptible to cell death in response to cellular stress or DNA damage.

12.6 Factors Associated with the p53 Protein Family

p53 expression is controlled at several levels including transcription, posttranscriptional modification, interactions with other proteins, and intracellular localization. The core control of p53-regulated degradation is ubiquitination by a number of ubiquitin ligases that target all three p53 family members. The ring domain E3 ubiquitin ligase, MDM2, is the critical negative regulator of p53 and is inhibited by p53 as its transcriptional target in a negative feedback loop [110]. MDM2 recognizes a short region of the p53 TAD and thereby interferes with p53 transcriptional activity, and interacts with the DBD region to promote p53 proteasomal degradation via ubiquitination.

MDMX (also known as MDM4) antagonizes p53 transcription without direct protein degradation, or heterodimerizes with MDM2 to augment p53 degradation via MDM2/MDM4 interaction with the C-terminal ring domains [111]. MDM2 does not promote p73 ubiquitination but induces its relocalization to subnuclear speckles, subsequently repressing p73 transcriptional activity [112]. The p53-MDM2 core circuit responds to multiple signaling pathways and can be regulated by various physiological clues, such as the Notch signaling pathway that binds to both MDM2 and p53 counteracting MDM2-dependent p53 ubiquitination and promoting p53 activity [113]. The activation of the Hedgehog pathway downregulates p53 by promoting MDM2 phosphorylation [114]. A recent report has suggested that the self-ubiquitination and ubiquitin ligase activity of MDM2 are inhibited by another interacting protein Enigma (a LIM domain protein), which subsequently promotes tumor-cell survival and chemoresistance by suppressing p53-mediated apoptosis [115].

In addition to MDM2, two other ubiquitin ligases, PIRH2 and COP1, are direct transcriptional targets of p53. Together with MDM2, these proteins are involved in a negative feedback loop to restore normal p53 levels through stimulus signals. TRIM24 (a ring-domain ubiquitin ligase) degrades p53 and FBXO45 (an F-box protein) promotes p73 degradation [96]. The crucial regulator of MDM2 is a tumor suppressor nucleolar protein, ARF, that interferes with the MDM2-p53 interaction to stabilize and activate p53 [116]. ARF binds and sequesters MDM2 in the nucleolus, then stabilizes nucleoplasmic p53. HAUSP induces DAXX-dependent MDM2 deubiquitination. The MDM2-DAXX-HAUSP complex is disrupted by RASSF1A protein, subsequently resulting in p53 stabilization [117]. p53 stabilization is also achieved by N-terminal phosphorylation at Ser-15 and Ser-20 which inhibits the interaction between p53 and MDM2 [118].

The p53 family proteins are also regulated by another ubiquitin ligase, WWP1, that induces ubiquitin-dependent proteasomal degradation and inhibits their transcriptional activity [119]. The NEDDS4-like ubiquitin ligase, ITCH, binds p63 and p73 and promotes their proteasomal degradation through its interaction with the C-terminal PY motif, which may be prevented by adaptor protein Yes-associated protein 1 (YAP-1) [120].

The transcriptional functions of the p53 family are coordinated through the transcriptional regulation of a wide array of cellular genes. The promoter selection of different sets of genes plays an integral part in determining the response to the p53 family members. The open status of chromatin induced by the induction of histone acetylation contributes to p53-dependent transactivation. The acetylase p300 / CREB-binding protein (CBP) and the pCAF acetyltransferases promote p53 transcriptional activity by catalyzing the acetylation of lysines within the p53 C-terminal region [118]. The modification of p53 by the JMY-STRA-PRMT5-p300/CBP complex activates $p21^{Waf1}$ to shift the p53 response toward cell-cycle arrest. Promyelocytic leukemia protein (PML) recruits p32-family proteins to subnuclear structures called PML nuclear bodies in which a number of common p53 interactors are found, including the acetylase p300, the protein kinase Hipk2, and the transcriptional repressor Daxx [96]. PML promotes the modification, stabilization,

and activation of the p53 family proteins. Axin, a component of the canonical WNT pathway, is another scaffolding protein involved in p53 regulation and with its interactors, Daxx and Hipk2. Axin contributes to cell fate determination in response to DNA damage by p53 phosphorylation at Ser-46 [121]. p63 and p73 also interact with the axin protein and control p53 modification by Hipk2 and Daxx.

The binding of specific interactors with the p53 family influences their target gene selection, resulting in the induction of cell-cycle arrest or apoptosis. The ASPP family proteins are involved with the proapoptotic regulation (BAX and PIG3) rather than the cell-cycle functions ($p21^{WAF1}$ or MDM2) of the p53 family proteins [52]. In contrast, the inhibitory protein iASPP inhibits proapoptotic gene activation but does not affect cell-cycle arrest after binding with p53. The iASPP protein also inhibits p73 and the dissociation of p73 from iASPP promotes p73-dependent apoptosis in p53-deficient cells [122]. As the cellular levels of ASPP are a crucial determinant of the apoptotic activation of the p53 pathway, the altered expression of ASPP or iASPP genes is frequently found in tumors [123]. Cabin1 also modulates p53 transcriptional activity on selected target gene promoters, such as Gadd45, $p21^{Waf1}$, Puma, Noxa, and MDM2 under normal conditions and binds to the p53 core DBD region to induce a transcriptional response after DNA damage [124]. Smad2 and Smad3, intracellular transducers of TGF-β signaling, interact with p53 family proteins to jointly control target gene activation by TGF-β and the p53 proteins [125].

The p53 family members are localized in the cytoplasm and p53 subcellular localization is regulated by several mechanisms, particularly ubiquitination. MDM2-mediated monoubiquitination induces nuclear export rather than p53 proteasomal degradation [126]. The E3 ligases WWP1 and MSL2 also induce p53 nuclear export, but the zinc-finger protein E4F1 does not induce p53 nuclear transport but stimulates its recruitment to chromatin and the activated expression of target genes [118]. Poly-ADP-ribose polymerase 1 (PARP-1) blocks p53 nuclear export and increases its transcriptional activities after DNA damage [127].

The most well-characterized nontranscriptional function of p53 is the induction of apoptosis through the mitochondrial pathway after DNA damage or oncogene activation. Monoubiquitinated p53 localizes to the mitochondria and interacts with the deubiquitinase HAUSP to form complexes with BCL2, BCL-xL, BAX, and BAK, thus promoting apoptosis [128]. MDMX can localize to the mitochondria and regulate p53 phosphorylation on Ser-46 to trigger the intrinsic apoptotic pathway via p53-BCL2 interactions [129]. Hipk2 also acts as one of the kinases responsible for p53 phosphorylation on Ser-46 and functions with MDMX as an interacting factor for p53 family members. BCL-2, BAX, BAK, and BCL-xL bind the DNA-binding domain of p53. p73 is cleaved by the caspase-3 and caspase-8 enzymes localized at the mitochondria that mediate TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, thereby inducing mitochondrial outer membrane permeabilization and apoptosis [130].

The regulation of autophagy is an emerging nonnuclear function of p53 involving the removal of damaged cytoplasmic organelles and the adaptation of cells to metabolic stress [131]. p53 transactivates genes that induce autophagy under stress

conditions, such as sestrin 1 and 2, TSC2, IGFBP3, PTEN, and DRAM. The degradation of cytoplasmic p53 by MDM2 and additional ubiquitin ligases (p300/CBP) also promotes autophagy after nutrient depletion or treatment with rapamycin [132].

A conserved microRNA (miRNA) family, miR-34, has been implicated in the p53 network as a direct transcriptional target of p53 [133]. DNA damage and oncogenic stress induce the expression of miR-34a, miR-34b, and miR-34c, thereby leading to apoptosis or cellular senescence. The miR-34a gene, residing at 1p36, is often lost in human tumors and 1p36 deletions involve a considerable number of genes [134]. The upregulation of miR-34a inhibits proliferation and activates a cell death pathway. The mir-34 loci are directly regulated by p53 interaction and the artificial delivery of miR-34 mimetics induces cell-cycle arrest (activation of the cdk inhibitor p21) or senescence, whereas the reduction of miR-34 function attenuates p53-mediated apoptosis. These different outcomes depend on the spectrum of miR-34 regulatory targets expressed in different cell types. Therefore, miR-34s participate in tumor suppression as a part of the p53 network. Reduced miR-34a expression is found in both neuroblastomas and pancreatic tumors [134], [135], and a reduced expression of miR-34b/34c is observed in non-small cell lung cancers (Bommer et al. 2007). Downregulation of miR-34a also promotes growth and tumor angiogenesis of HNSCCs and is associated with a poor prognosis in cisplatin-treated sinonasal squamous cell carcinoma (SCC) patients [136], [137].

The cellular loss of p53 abrogates a number of crucial regulatory networks that contribute to cancer. p53 mutants bind p63 and p73 and inhibit their functions and those of other common interacting proteins, associated with the cancer phenotype [138]. The functional loss and deregulated expression of some p53 is frequently seen in different cancers and transformed cells bearing wild-type p53 but showing increased activity of p53 interactors such as MDM2, p14ARF, iASPP, and ChK2, etc. These alterations have a significant impact on cancer prognosis and therapeutic response. The recovery of p53 and its interactor functions is a potentially promising strategy to overcome cancer therapeutic resistance.

12.7 Assessing p53 Status and *TP53* Abnormalities as Cancer Biomarkers

The accumulation of p53 protein in HNC and tumors has been commonly used as a surrogate biomarker for *TP53* mutations. As discussed above, many mutations stabilize the protein and slow degradation. As p53 is not a stable protein in nontumor tissues of patients or mice with germline *TP53* mutations, its overexpression is typically associated with tumor development. Immunohistochemical (IHC) analysis of p53 has been explored to assist with the clinical diagnosis, prognosis, and treatment of cancer. However, using expression of p53 to assess gene status may mask nonsense mutations, insertions, deletions, or splicing junction mutations that account for up to 30 % of somatic *TP53* mutations [139]. Although, tumors with p53 null mutations have negative IHC results, these can be more aligned with clinical outcomes than

a positive IHC result [140]. Moreover, wild-type p53 protein can be accumulated in response to an inflammatory microenvironment or even as a result of senescence [141], [142]. Nonetheless, availability of routine histopathologic assessment and scoring in a pathologic laboratory is the obvious advantage of p53 immunostaining. Evaluation of MDM2 and p21^{waf1} combined with p53 staining in tumors may provide an approach to detect functional inactivation and transcriptional activity by discriminating functional and nonfunctional p53 in the absence of a *TP53* mutation [143]. p53 and MDM2 staining also provides information on the p53 phenotype and is complementary to *TP53* genotyping data. When used as one of a panel of biomarkers, p53 staining may provide important clinical information. For example, p53, EGFR, HPV tilter, and Bcl-xL, and smoking have been explored as combined biomarkers of a patient's response to therapy and survival in oropharyngeal cancer. Low p53 expression combined with high Bcl-xL and EGFR expression is associated with poor outcomes after organ-sparing therapy [136]. However, there has been much evidence that protein expression analysis alone cannot achieve an accurate picture of the p53 status.

The gold standard method for *TP53* mutation analysis of tumors is DNA sequencing. Initial mutational screening may be achieved by gel-based mutation assays, such as SSCP or PCR-RFLP. These techniques include the processes of *TP53* amplification and enzymatic restriction of exon-specific PCR fragments that act as an indicator for the presence of mutations. Direct sequencing methods for altered areas then generate information on the *TP53* status. Many mutation analyses for *TP53* have been limited to exons 5–8 in which most p53 mutations are found, and thus fail to detect mutations outside this portion of the gene. This issue may be solved with more targeted high-throughput DNA sequencing methods to gather nucleotide-level information about *TP53* and other tumor suppressor genes in cancer samples [144].

New technologies using spotted oligonucleotide arrays have increased the sensitivity and specificity of mutation detection. The p53 GeneChip assay is a rapid and accurate approach to detecting single base-pair substitutions and deletions in the *TP53* coding sequence [145], [146]. The GeneChip assay is designed for high-throughput detection of mutations in *TP53* exons 2–11. After one-round of amplification of *TP53* exons and fragmentation, DNA amplicons are hybridized to oligonucleotide probes to detect base-pair mutations and deletions for each nucleotide within the full *TP53*-coding sequence. The reported specificity of both the p53 GeneChip assay and direct sequencing for detecting p53 mutations are comparable (98 –100 %) (145). The GeneChip technique as a high-throughput sequencing method for *TP53* may provide valuable new data for cancer mutational spectra in population-based studies [147].

Abnormal p53 expression and *TP53* mutations have been associated with poor survival outcomes or poor responses to therapy. *TP53* mutations are also associated with an increased risk of locoregional failure in HNSCC patients after radiotherapy [148]. Nonetheless, a lack of prospective studies, the heterogeneity in different tumor types, variation in experimental design, and the inherent complexity of the p53 pathway have lessened the clinical reliability of p53 status for prediction of cancer patient outcomes. Data on mutations and prognoses extracted from the IARC *TP53*

database reveal an association between p53 mutations and poor survival outcomes for cancers of the head and neck, breast, liver, hematopoietic and lymphoid systems but not for cancers of the pancreas, prostate gland, rectum, and stomach (http://www-p53.iarc.fr).

Recent meta-analysis has systematically reviewed p53 as a prognostic biomarker of overall survival (OS) and disease-free survival (DFS) in patients with SCC of the oral cavity, oropharynx, larynx, and hypopharynx [149]. A statistically significant hazard ratio (HR) for OS was found only in the oral cavity (pooled HR = 1.48) and for DFS in the oral cavity (pooled HR = 1.47) and the oropharynx (pooled HR = 0.45). Because large heterogeneity exists across studies of p53 and cancer survival, there is inconclusive evidence on the prognostic value of p53 in SCC patients in the four main anatomical subsites of the head and neck. A prospective, multicenter study, however, has shown a significant association between *TP53* mutation status and survival for SCC of the head and neck in 420 patients treated surgically [32]. Mutations of *TP53* exons 2–11 in tumor tissue detected through the GeneChip assay and Surveyor denaturing high-performance liquid chromatography (DHPLC) were examined for the presence of disruptive or nondisruptive mutations. Nonconservative mutations located in the core DNA-binding domain (L2–L3 region), or stop codons in any region were defined as disruptive, and others as nondisruptive mutations. p53 mutations showed a significant association with a shorter OS compared with wild-type p53 tumors, and disruptive mutations in p53 had a stronger association with poor survival than nondisruptive mutations (Fig. 12.5). These results were also supported by an orthotopic murine model of oral tongue cancer examining disruptive *TP53* mutations and aggressive tumor characteristics [150]. One mechanism for the prognostic impact of disruptive p53 mutation may be inhibition of radiation-induced cellular senescence, as SCC cell lines with disruptive p53 mutation have been found to be significantly more radiation resistant, and this effect was potentiated by the mitochondrial agent, metformin [40]. Another recent study has shown that a truncating *TP53* mutation, that is nondisruptive mutation, is associated with a poor prognosis [151]. Additional studies are required to firmly establish the prognostic importance of p53 status for HNC survival using prospective patient data.

The data obtained from studies examining the role of *TP53* mutation as a predictor of therapeutic response are complex. In an NCI60 human tumor cell line anticancer drug screen, p53 mutants were found to be associated with an average 2.6-fold lower sensitivity to 66 of 132 chemotherapeutic agents including alkylating agents, topoisomerase I and II inhibitors, and RNA/DNA antimetabolites [152]. *TP53* mutations were also shown to be a biomarker of the responsiveness to carboplatin or gemcitabine treatment in NSCLC [153] and to cisplatin in HNSCC [154]. Mutation of p53 within its C-terminal region may cause a loss of nuclear p53 signal that correlates with cisplatin resistance in HNSCC cell lines. These results could help to identify nonresponders to specific agents before initiating chemotherapy. In contrast, it has been also reported that tumors with p53 overexpression are more responsive to cisplatin than p53-null lesions [155]. This variation in chemosensitivity may result from a common sequence polymorphism of *TP53* encoding either arginine or proline at amino acid position 72. The Pro72 variant induces apoptosis significantly better than

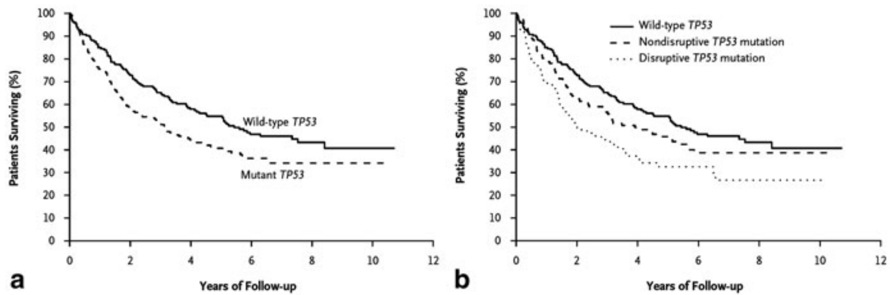

Fig. 12.5 Overall survival outcomes for HNSCC patients according to their p53 mutational status and category. **a** A prospective multicenter study showed that 196 patients with wild-type *TP53* had a longer median survival than 224 patients harboring mutant *TP53* (5.4 years vs. 3.2 years). **b** Overall survival of 85 patients with disruptive *TP53* mutations was poorer than that of 139 patients with nondisruptive *TP53* mutations. Disruptive mutations were defined as nonconservative mutations located in the core DNA-binding domain (L2–L3 regions) of p53, or stop codons in any region. Nondisruptive mutations were defined as conservative or nonconservative mutations outside of the L2–L3 region. (Reproduced with permission from New England Journal of Medicine [32])

the Arg72 variant by stimulating a higher release of cytochrome c after localization to the mitochondria [156]. A clinical trial has also shown that the Arg72 polymorphism is associated with a poor response to cisplatin-based chemoradiotherapy for advanced HNC [87]. The effect of p53 polymorphisms on the individual responsiveness to cancer therapy may be explained by the fact that Arg72 mutants efficiently inhibit p73 and act as dominant-negative inhibitors of both p53 and p73. The p53 mutants V143A and R175H block the response of many tumors with an Arg72 polymorphism to many anticancer agents by inhibiting p73 more efficiently than the Pro72 forms. Other genetic events may also affect functional p53 pathways, thereby suppressing apoptotic mechanisms and promoting continued tumor growth. Loss of the Bcl2 family, caspase-encoding genes and sensitivity to extrinsic apoptotic stimuli may engender resistance to the host immune response and apoptosis [157]. Tumors harboring different defects in these pathways typically show resistance to chemotherapy or radiotherapy. However, there is still a lack of conclusive evidence for a role of *TP53* mutations in the response to first-line chemotherapy or radiotherapy. This will need to be elucidated in a prospective large cohort of HNC patients.

Surgery remains the principal treatment modality for many cancer patients. Routine clinical evaluation and microscopic examination has been utilized for the identification of residual tumors at the surgical margins following tumor resection. Even after excising a tumor with a generous margin of normal tissue, tumors can recur despite microscopically free margins, and second tumors can develop at other sites in patients effectively treated for an index tumor. Locoregional recurrence remains the most common cause of surgical treatment failures for patients with HNSCC. New diagnostic approaches are required to provide better mapping of this elusive cancer spread and permit accurate assessment of surgical margins by providing molecular evidence of residual tumors. Histologically normal mucosal margins may contain genetically altered cells including *TP53* mutations, promoter hypermethylation and

proto-oncogene overexpression, etc. Because the *TP53* sequence is frequently mutated in HNCs, its mutations can be used as a target for tumor cell detection in surrounding normal tissues or surgical margins without the need to microscopically detect residual tumors on pathologic examination. This approach is based on finding the same signature *TP53* mutation in a tumor and in histologically negative surgical margins that can cause tumor recurrence even after successful radical resection.

Initial molecular analyses of HNSCC surgical margins showed very promising results. In a landmark study, margins harboring mutations of the p53 gene were positive in 13 of 25 HNSCC patients, of whom five had tumor local recurrence, as compared with 12 patients with a negative margin who showed no recurrence [30]. This early molecular study employed a phage plaque assay containing p53 gene fragments encompassing exons 5–9 that were isolated from tumors to probe DNA from surgical margins. Margin DNA was amplified in phage, plated on a bacterial lawn, transferred to filters, and probed with radiolabeled oligonucleotides. The residual tumor cells harboring a p53 mutation were estimated for mucosal and deep surgical margins by comparison with a primary tumor positive control. These results have been further supported by a prospective study using the same phage plaque assay technique [158]. This test could aid clinicians in the delineation of a tumor resection area and help with the planning of postoperative adjuvant therapy. However, it is technically cumbersome and expensive to perform. A first generation test that requires 4–6 weeks to generate results is now commercially available to clinicians but more rapid approaches are needed for proper intraoperative decision making. The time and technical limitations of this assay have been improved by a real-time semiquantitative gap ligase chain reaction for the detection of *TP53* point mutations, with sensitivity comparable to that found for the phage plague assay for tumor cell detection [159]. This method used two sets of primers spanning *TP53* mutant sequences and a complimentary primer bound to the mutated sequence and fluorescent labeled probe to allow for quantitative real-time analysis. It can detect small numbers of tumor cells in a background with a large excess of wild-type DNA. Positive margins included tissue samples with a tumor/margin DNA ratio of as low as 1:1,000. Further advances have been made possible by the Ligamp *TP53* assay which provides sensitive and quantifiable detection of mutant *TP53* in histologically normal margins [160]. This approach can assess single nucleotide genetic alterations with a sensitivity of one mutant species among 10,000 wild-type molecules. The study of Poeta and coworkers showed that the sensitivity and specificity for the prediction of tumor recurrence in 95 patients with common *TP53* mutations were 60 and 68 %, respectively. However, no statistical significance of positive margin predictive of cancer specific or OS was observed in this cohort. Clinical trials using these molecular diagnostics are currently ongoing to determine whether molecular detection of residual tumors can identify subgroups at high risk of locoregional recurrence.

Precancerous fields in the upper aerodigestive tract are known to be at risk of developing a second tumor [161]. It is also well known that some areas of leukoplakia or erythroplakia progress to invasive HNC. Many of these premalignant lesions harbor the genetic aberrations associated with tumor progression [162]. Molecular

diagnostic approaches to identify the presence of cells harboring *TP53* mutations in tumor-remote mucosa may help identify patients at risk for second cancers. The presence of a *TP53* mutation in histologically normal mucosa or precursor lesions may provide information on the risk of progression to cancer [163]. In addition, multiple molecular markers including *TP53* mutations may be useful in determining the clonal origin of multiple tumors or tumor spread in synchronous cancers [164]. This molecular approach helps identify whether a second tumor is a recurrence of a primary index tumor or a new primary lesion. Concordant aberrations of paired tumors (microsatellite allele, *TP53* mutation, and female X chromosomal analyses) suggest recurrence of an index tumor [165]. This highlights the need to treat precursor lesions in the affected field as well as addressing visible tumors.

12.8 Restoration of p53 Function: Therapeutic Exploitation of p53

Increasing numbers of specific gene mutations in human cancers have become evident, and the subsequent development of agents targeting these molecular abnormalities holds promise for valuable advances in cancer therapy. Cancer therapies that target specific mutant genes are proving to be highly effective and selectively toxic to some tumor types. Currently, around 22 million people are living with a diagnosis of cancer worldwide, of whom more than 50 % have mutations of p53 gene and the other 50 % have no *TP53* mutation but inactivation of the p53 pathway. Insights into p53 biology have generated mouse models that support the potential value of activating p53 as an anticancer therapy. Mice carrying an extra copy of *Trp53* have enhanced p53 activation upon stress and demonstrate both protection from cancer and delayed aging under normal p53 regulation [166]. In addition, when p53 in the mouse was genetically engineered to "switch off" to allow tumor formation and then "switch on" to test this impact on established tumors, substantial tumor regression via the reinstatement of p53 expression was observed [167]–[169]. Therefore, the activation of p53 is a prime potential target for anticancer drug development. Current trials targeting p53 function include *TP53*-gene therapies, p53-based vaccines, and small molecules that activate p53-dependent transcription and modulate p53 function.

Viral vector-mediated *TP53* gene transfer has been used to treat advanced and recurrent HNSCC. Oncolytic virus locally injected into tumors infects and spreads a functional copy of *TP53* among the tumor cells. Because adenoviruses infect and transduce both dividing and nondividing cells with high gene transfer efficiencies, these have been used to enhance replication capacity in tumor cells with p53 dysfunction. ONYX-015 is an adenovirus lacking the E1B 55-kDa gene, the product of which binds and inactivates p53, and is engineered to selectively replicate within and lyse *TP53*-mutant tumor cells but not normal cells [170]. Intratumoral administration of ONYX-015 has been extensively tested in clinical trials for systemic safety and therapeutic efficacy when used to combat HNC in a combination with or without standard chemotherapeutic agents [171], [172]. However, therapeutic effects

following injection have been limited to partial regression in a few cases and subsequent development of ONYX-015 was discontinued. Genedicine (Shenzhen SiBiono GeneTech, Shenzhen, China), an adenovirus engineered to express wild-type p53, was approved by the Chinese FDA to treat HNSCC and various forms of cancer. Advexin®, a *TP53*-producing adenovirus equivalent to Genedicine, has not been approved in the USA or Europe. Extensive clinical trials of these materials showed clear responses in some patients but a general failure to achieve adequate therapeutic efficacy in human cancers [173]. The potential applications of these agents might involve patients who have unresectable or recurrent HNSCC and require palliative management. The interest in p53-based tumor vaccination will be revived if improved vectors are developed [174].

An advanced understanding of regulatory T cells and the signaling pathways related to the immune response has led to the recent development of cancer vaccines. An antibody against CTL4 neutralizes its suppression of the immune response; clinical trials of ipilimumab for the treatment of melanoma have shown remarkable long-term cures in 35 % of patients [175]. High tumor-specific expression levels of *TP53* and its frequent mutation in human cancers suggests that p53 may be perceived by the host immune system as a target antigen. Immunization with large peptides derived from p53 has been attempted for some time in cancer patients who make antibodies to p53, but the adequacy of response is still debated. A number of clinical trials using synthetic peptide mixtures derived from p53 revealed insufficient potency to result in a clinical benefit due to the failure of T-helper cells to produce key cytokines and a p53-specific T-helper response. Both DNA-based and dendritic cell-delivered p53 vaccines promote a more correctly polarized and stronger T-cell response. INGN-225 (Introgen), a dendritic cell vaccine with p53-modified adenoviral transduction, induces a significant host immune response that sensitizes tumors to subsequent chemotherapy [176]. p53-based immunotherapy awaits validation of safety and efficacy in the treatment of HNC.

In the field of chemical biology, an entirely different set of strategies have centered on the development of nongenotoxic low-molecular-mass compounds that activate p53 tumor suppressive function in human cancers. Reverse or forward chemical genetic approaches have led to the identification of numerous small molecules that selectively kill tumor cells by activating wild-type or mutant p53. This biochemical screening has made progress along several distinct lines. With a thorough understanding of the p53-MDM2 interaction, inhibitors of this major negative regulator of p53 have been developed that show restoration of p53 transcriptional function and clear antitumor activity with no detectable genotoxicity [45]. In approaches that target mutant p53, small molecules bind to either full-length p53 or the core DNA-binding domain of mutant p53, therefore restoring its activity and inhibiting tumor growth [177], [178]. Trials testing the efficacy of such compounds are actively ongoing at a preclinical and clinical stage.

Disruption of the p53-MDM2 interaction restores p53 function and sensitizes tumors to radiotherapy or chemotherapy. Selective inhibitors of MDM2 currently being investigated include JNJ-26854165 (Johnson & Johnson, New Brunswick,

NJ), RG7112 (F. Hoffmann-La Roche, Basel, Switzerland), MI-219 (Asenta Therapeutics, Malvern, PA), PXN727 and PXN822 (Priaxon, Munich, Germany), nutlin (Roche), and RITA (reactivation of p53 and induction of tumor cell apoptosis, Aprea, Solna, Sweden) [179]. These small molecules compete with MDM2 for its p53-binding pocket.

Nutlins are the first reported small-molecule MDM2 antagonists identified from a class of *cis*-imidazoline compounds by Vassilev and colleagues [45], [180]. The nutlins bind to the p53 pocket of MDM2 and mimic the molecular interactions between MDM2 and the three residues of the helical region of the p53 TAD (Phe19, Trp23, and Leu26) critical for its binding to p53. This small molecule selectively induces a growth-inhibiting state called senescence via the arrest of the G1 and G2 phases of the cell cycle or apoptosis at micromolar concentrations in cancer cells that contain normal or wild-type p53. Nutlin-3 is the compound most commonly used in anticancer studies and stabilizes p53 by inhibiting its interaction with MDM2. Nutlin-3 potently induces apoptosis in hematologic cancer cells including AML, ALL, B-cell CLL, and myeloma and causes tumor shrinkage with few toxic side-effects [181]. Nutlin-3 has also been shown to operate synergistically with a wide variety of cytotoxic agents and radiation treatments in preclinical studies [182, 183]. The clinical Phase I trial for nutlin is currently progressing in patients with hematologic neoplasms [179]. A small molecule RITA has also been identified in a phenotypic screen for molecules that suppress cancer cells containing wild-type p53 [184]. RITA fills the MDM2 binding pocket of p53, i.e., residues 1–63 of the TAD, and induces a conformational change that reduces the interaction between p53 and MDM2. RITA also inhibits the binding of two other p53 inhibitors, iASPP and Parc. RITA induces p53-dependent apoptosis in different types of cancer cells by significantly affecting genes involved in apoptotic pathway, whereas nultin-3 induces mainly cell-cycle arrest genes [185]. RITA was also recently shown to induce apoptosis of HNC cells and enhance the cytotoxicity of cisplatin both in vitro and in vivo [186].

The core DNA-binding domain of p53 is rather unstable, leading to partial unfolding at a melting temperature of 44°C, and mutations in this region thermally destabilize the p53 protein and abrogate its DNA-binding activity [187]. The inherent instability of the p53 DNA-binding domain has led to the new pharmacologic approaches that seek to stabilize the binding activity and restore the function of different mutant p53 proteins by shifting the defect toward an active, properly folded form. Peptide 46, a short synthetic peptide derived from the p53 C-terminus, has provided proof of principle for mutant p53 rescue and the subsequent induction of p53-dependent apoptosis in tumors with mutant *TP53* [177]. Further efforts have been made to identify low-molecular-weight compounds for reactivation of mutant p53 by using either biochemical or phenotypic screens of chemical libraries. Representative small molecules that restore p53 function in mutant *TP53*-carrrying tumor cells include CP-31398 (Pfizer, New York, NY), PRIMA-1 (Aprea, Solna, Sweden), MIRA-1 (Aprea), STIMA-1 (Aprea), and RETRA.

CP-31398 stabilizes the active conformation of p53 and promotes its functional activity in cancer cells with either mutant or wild-type p53 by preventing its ubiquitination without blocking the p53-MDM2 interaction [188]. The exact mechanism

of activity of this small molecule is unknown. However, CP-31398 activates a small set of known p53 target genes in mutant p53-carrying cancer cells and suppresses spontaneous tumors in immunocompetent mouse models or inhibits the growth of xenograft tumors in immunodeficient mice [178], [189], [190]. CP-31398 induces the classic proapoptotic p53 target genes *PUMA* and *BAX*, the mitochondrial translocation of p53, and the downregulation of cyclins A, B1, D1, and E, indicating a restoration of the transcriptional activity of p53 [189]. A cell-based screen from a library of diverse compounds has uncovered a compound known as PRIMA-1 that directly interacts with mutant p53 [44]. PRIMA-1 restores the sequence-specific DNA binding and transactivation activity of mutant p53 proteins *in vitro* and in living cells. *In vivo* studies have further revealed antitumor effects of this molecule in human tumor xenograft models of different types with no apparent toxicity in normal tissues [44]. PRIMA-1 induces caspase-dependent apoptosis in a transcription-independent manner via its interaction with Bcl-2 family proteins in the cytoplasm [191]. This small molecule is now in clinical Phase I/II trials (Aprea, Solna, Sweden) [179]. Another small molecule, RETRA, that was recently discovered in a cell-based screen, reactivates p53 function specifically in mutant p53-bearing cancer cells [89]. The tumor suppressive mechanisms of RETRA also include the release of p73 from the inhibitory complex with mutant p53 and subsequent induction of p73 transcriptional target genes. PRIMA-1 can also release active p73. Another small molecule, p53R3, was identified by a biochemical screening of a chemical library [192] and restores the *in vitro* and *in vivo* DNA-binding activity of p53 with mutations at residues 175, 273, and, to some extent, 248 and 237, resulting in the p53-dependent induction of target genes involved in growth arrest (p21 ad GADD45) and of proapoptotic targets (PUMA, DR5 and CD95 L). In addition, a recent study showed that a small molecule targeting the CH1 domain of p300, CH1iB, reactivates p53 and potentiates the anticancer activity of *cis*-platinum in HPV16-positive HNSCC cells by blocking the association between HPV E6 and p300 [180]. However, small molecules targeting mutant p53 proteins have been rarely examined in the context of HNC [193]. This needs to be examined in future studies.

References

1. Cancer LDP. p53, guardian of the genome. Nature. 1992;358(6381):15–6.
2. Kress M, May E, Cassingena R, May P. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J Virol. 1979;31(2):472–83. PMCID: 353470.
3. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell. 1979;17(1):43–52.
4. Melero JA, Stitt DT, Mangel WF, Carroll RB. Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. Virology. 1979;93(2):466–80.
5. Smith AE, Smith R, Griffin B, Fried M. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell. 1979;18(4):915–24.
6. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature. 1979;278(5701):261–3.

7. DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci U S A. 1979;76(5):2420–4. PMCID: 383613.
8. Sarnow P, Sullivan CA, Levine AJ. A monoclonal antibody detecting the adenovirus type 5-E1b-58Kd tumor antigen: characterization of the E1b-58Kd tumor antigen in adenovirus-infected and -transformed cells. Virology. 1982;120(2):510–7.
9. Wolf D, Rotter V. Inactivation of p53 gene expression by an insertion of Moloney murine leukemia virus-like DNA sequences. Mol Cell Biol. 1984;4(7):1402–10. PMCID: 368923.
10. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, et al. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science. 1989;244(4901): 217–21.
11. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell. 1989;57(7):1083–93.
12. Lavigueur A, Maltby V, Mock D, Rossant J, Pawson T, Bernstein A. High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol Cell Biol. 1989;9(9):3982–91. PMCID: 362460.
13. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992;356(6366):215–21.
14. Harvey M, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet. 1993;5(3):225–9.
15. Li FP, Fraumeni JF Jr. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med. 1969;71(4):747–52.
16. Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. 1990;250(4985):1233–8.
17. Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature. 1990;348(6303): 747–9.
18. Varley JM. Germline *TP53* mutations and Li-Fraumeni syndrome. Hum Mutat. 2003;21(3):313–20.
19. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature. 1993;362 (6423):857–60.
20. Wu X, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 1993;7(7A):1126–32.
21. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997;387(6630):296–9.
22. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature. 1997;387(6630):299–303.
23. Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, Sherr CJ. Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad Sci U S A. 1998;95(14):8292–7. PMCID: 20969.
24. Shvarts A, Steegenga WT, Riteco N, van Laar T, Dekker P, Bazuine M, et al. MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J. 1996;15(19): 5349–57. PMCID: 452278.
25. Linares LK, Hengstermann A, Ciechanover A, Muller S, Scheffner M. HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proc Natl Acad Sci U S A. 2003;100(21):12009–14. PMCID: 218704.
26. Wade M, Wahl GM. Targeting Mdm2 and Mdmx in cancer therapy: better living through medicinal chemistry? Mol Cancer Res. 2009;7(1):1–11. PMCID: 2629357.
27. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.

28. Sturgis EM, Cinciripini PM. Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer. 2007;110(7):1429–35.
29. Pai SI, Westra WH. Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. Ann Rev Pathol. 2009;4:49–70.
30. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med. 1995;332(7):429–35.
31. Gonzalez MV, Pello MF, Lopez-Larrea C, Suarez C, Menendez MJ, Coto E. Loss of heterozygosity and mutation analysis of the p16 (9p21) and p53 (17p13) genes in squamous cell carcinoma of the head and neck. Clin Cancer Res. 1995;1(9):1043–9.
32. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, et al. *TP53* mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357(25):2552–61. PMCID: 2263014.
33. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63(6):1129–36.
34. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993;75(4):817–25.
35. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995;80(2):293–9.
36. Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature. 2009;458(7242):1127–30. PMCID: 2814168.
37. Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, et al. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med. 1996;2(7):811–4.
38. O'Shea D, O'Riain C, Taylor C, Waters R, Carlotti E, Macdougall F, et al. The presence of *TP53* mutation at diagnosis of follicular lymphoma identifies a high-risk group of patients with shortened time to disease progression and poorer overall survival. Blood. 2008;112(8):3126–9. PMCID: 2954748.
39. Perrone F, Bossi P, Cortelazzi B, Locati L, Quattrone P, Pierotti MA, et al. *TP53* mutations and pathologic complete response to neoadjuvant cisplatin and fluorouracil chemotherapy in resected oral cavity squamous cell carcinoma. J Clin Oncol. 2010;28(5):761–6.
40. Skinner HD, Sandulache VC, Ow TJ, Meyn RE, Yordy JS, Beadle BM, et al. *TP53* disruptive mutations lead to head and neck cancer treatment failure through inhibition of radiation-induced senescence. Clin Cancer Res. 2012;18(1):290–300. PMCID: 3251726.
41. Peng Z. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther. 2005;16(9):1016–27.
42. Senzer N, Nemunaitis J. A review of contusugene ladenovec (Advexin) p53 therapy. Curr Opin Mol Ther. 2009;11(1):54–61.
43. Kirn D, Hermiston T, McCormick F. ONYX-015: clinical data are encouraging. Nat Med. 1998;4(12):1341–2.
44. Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med. 2002;8(3):282–8.
45. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science. 2004;303(5659):844–8.
46. Joerger AC, Fersht AR. Structural biology of the tumor suppressor p53. Ann Rev Biochem. 2008;77:557–82.
47. Teufel DP, Freund SM, Bycroft M, Fersht AR. Four domains of p300 each bind tightly to a sequence spanning both transactivation subdomains of p53. Proc Natl Acad Sci U S A. 2007;104(17):7009–14. PMCID: 1855428.
48. Toledo F, Lee CJ, Krummel KA, Rodewald LW, Liu CW, Wahl GM. Mouse mutants reveal that putative protein interaction sites in the p53 proline-rich domain are dispensable for tumor suppression. Mol Cell Biol. 2007;27(4):1425–32. PMCID: 1800716.

49. Bullock AN, Henckel J, Fersht AR. Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy. Oncogene. 2000;19(10):1245–56.
50. Kitayner M, Rozenberg H, Kessler N, Rabinovich D, Shaulov L, Haran TE, et al. Structural basis of DNA recognition by p53 tetramers. Mol Cell. 2006;22(6):741–53.
51. Tang Y, Luo J, Zhang W, Gu W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. Mol Cell. 2006;24(6):827–39.
52. Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, et al. ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell. 2001;8(4):781–94.
53. Mateu MG, Fersht AR. Nine hydrophobic side chains are key determinants of the thermodynamic stability and oligomerization status of tumour suppressor p53 tetramerization domain. EMBO J. 1998;17(10):2748–58. PMCID: 1170615.
54. Brokx RD, Bolewska-Pedyczak E, Gariepy J. A stable human p53 heterotetramer based on constructive charge interactions within the tetramerization domain. J Biol Chem. 2003;278(4):2327–32.
55. Rustandi RR, Baldisseri DM, Weber DJ. Structure of the negative regulatory domain of p53 bound to S100B(betabeta). Nat Struct Biol. 2000;7(7):570–4.
56. Sheng Y, Saridakis V, Sarkari F, Duan S, Wu T, Arrowsmith CH, et al. Molecular recognition of p53 and MDM2 by USP7/HAUSP. Nat Struct Mol Biol. 2006;13(3):285–91.
57. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997;90(4):595–606.
58. McKinney K, Mattia M, Gottifredi V, Prives C. p53 linear diffusion along DNA requires its C terminus. Mol Cell. 2004;16(3):413–24.
59. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on *TP53* mutation patterns and tumor phenotype: lessons from recent developments in the IARC *TP53* database. Hum Mutat. 2007;28(6):622–9.
60. Kato S, Han SY, Liu W, Otsuka K, Shibata H, Kanamaru R, et al. Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. Proc Natl Acad Sci U S A. 2003;100(14):8424–9. PMCID: 166245.
61. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science. 1994;265(5170):346–55.
62. Ang HC, Joerger AC, Mayer S, Fersht AR. Effects of common cancer mutations on stability and DNA binding of full-length p53 compared with isolated core domains. J Biol Chem. 2006;281(31):21934–41.
63. Joerger AC, Ang HC, Fersht AR. Structural basis for understanding oncogenic p53 mutations and designing rescue drugs. Proc Natl Acad Sci U S A. 2006;103(41):15056–61. PMCID: 1635156.
64. Joerger AC, Ang HC, Veprintsev DB, Blair CM, Fersht AR. Structures of p53 cancer mutants and mechanism of rescue by second-site suppressor mutations. J Biol Chem. 2005;280(16):16030–7.
65. Zhang W, Guo XY, Hu GY, Liu WB, Shay JW, Deisseroth AB. A temperature-sensitive mutant of human p53. EMBO J. 1994;13(11):2535–44. PMCID: 395127.
66. Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, et al. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. N Engl J Med. 1995;332(11):712–7.
67. Cabelguenne A, Blons H, de Waziers I, Carnot F, Houllier AM, Soussi T, et al. p53 alterations predict tumor response to neoadjuvant chemotherapy in head and neck squamous cell carcinoma: a prospective series. J Clin Oncol. 2000;18(7):1465–73.
68. Alsner J, Sorensen SB, Overgaard J. *TP53* mutation is related to poor prognosis after radiotherapy, but not surgery, in squamous cell carcinoma of the head and neck. Radiother Oncol. 2001;59(2):179–85.
69. Blons H, Laurent-Puig P. *TP53* and head and neck neoplasms. Hum Mutat. 2003;21(3):252–7.

70. Ko Y, Abel J, Harth V, Brode P, Antony C, Donat S, et al. Association of CYP1B1 codon 432 mutant allele in head and neck squamous cell cancer is reflected by somatic mutations of p53 in tumor tissue. Cancer Res. 2001;61(11):4398–404.
71. Temam S, Flahault A, Perie S, Monceaux G, Coulet F, Callard P, et al. p53 gene status as a predictor of tumor response to induction chemotherapy of patients with locoregionally advanced squamous cell carcinomas of the head and neck. J Clin Oncol. 2000;18(2):385–94.
72. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92(9):709–20.
73. Westra WH, Taube JM, Poeta ML, Begum S, Sidransky D, Koch WM. Inverse relationship between human papillomavirus-16 infection and disruptive p53 gene mutations in squamous cell carcinoma of the head and neck. Clin Cancer Res. 2008;14(2):366–9.
74. Wong RH, Du CL, Wang JD, Chan CC, Luo JC, Cheng TJ. XRCC1 and CYP2E1 polymorphisms as susceptibility factors of plasma mutant p53 protein and anti-p53 antibody expression in vinyl chloride monomer-exposed polyvinyl chloride workers. Cancer Epidemiol Biomarkers Prev. 2002;11(5):475–82.
75. Tabor MP, Brakenhoff RH, van Houten VM, Kummer JA, Snel MH, Snijders PJ, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res. 2001;7(6):1523–32.
76. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991;253(5015):49–53.
77. Shaulsky G, Goldfinger N, Rotter V. Alterations in tumor development in vivo mediated by expression of wild type or mutant p53 proteins. Cancer Res. 1991;51(19):5232–7.
78. Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer. 2009;9(10):701–13.
79. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res. 2000;60(24):6788–93.
80. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med. 1995;1(10):1029–34.
81. Reich NC, Levine AJ. Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. Nature. 1984;308(5955):199–201.
82. Barak Y, Juven T, Haffner R, Oren M. Mdm2 expression is induced by wild type p53 activity. EMBO J. 1993;12(2):461–8. PMCID: 413229.
83. Rotter V. p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. Proc Natl Acad Sci U S A. 1983;80(9):2613–7. PMCID: 393877.
84. Iggo R, Gatter K, Bartek J, Lane D, Harris AL. Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet. 1990;335(8691):675–9.
85. Lukashchuk N, Vousden KH. Ubiquitination and degradation of mutant p53. Mol Cell Biol. 2007;27(23):8284–95. PMCID: 2169174.
86. Muller P, Hrstka R, Coomber D, Lane DP, Vojtesek B. Chaperone-dependent stabilization and degradation of p53 mutants. Oncogene. 2008;27(24):3371–83.
87. Bergamaschi D, Gasco M, Hiller L, Sullivan A, Syed N, Trigiante G, et al. p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell. 2003;3(4):387–402.
88. Marin MC, Jost CA, Brooks LA, Irwin MS, O'Nions J, Tidy JA, et al. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat Genet. 2000;25(1):47–54.
89. Kravchenko JE, Ilyinskaya GV, Komarov PG, Agapova LS, Kochetkov DV, Strom E, et al. Small-molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway. Proc Natl Acad Sci U S A. 2008;105(17):6302–7. PMCID: 2327210.
90. Zhang Y, Sturgis EM, Huang Z, Zafereo ME, Wei Q, Li G. Genetic variants of the p53 and p73 genes jointly increase risk of second primary malignancies in patients after index squamous cell carcinoma of the head and neck. Cancer. 2012;118(2):485–92. PMCID: 3184342.

91. Kim E, Deppert W. Transcriptional activities of mutant p53: when mutations are more than a loss. J Cell Biochem. 2004;93(5):878–86.
92. Matas D, Sigal A, Stambolsky P, Milyavsky M, Weisz L, Schwartz D, et al. Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J. 2001;20(15):4163–72. PMCID: 149170.
93. Scian MJ, Stagliano KE, Anderson MA, Hassan S, Bowman M, Miles MF, et al. Tumor-derived p53 mutants induce NF-kappaB2 gene expression. Mol Cell Biol. 2005;25(22):10097–110. PMCID: 1280285.
94. Song H, Hollstein M, Xu Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol. 2007;9(5):573–80.
95. Stiewe T. The p53 family in differentiation and tumorigenesis. Nat Rev Cancer. 2007;7(3):165–8.
96. Collavin L, Lunardi A, Del Sal G. p53-family proteins and their regulators: hubs and spokes in tumor suppression. Cell Death Differ. 2010;17(6):901–11.
97. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999;398(6729):708–13.
98. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999;398(6729):714–8.
99. Flores ER, Sengupta S, Miller JB, Newman JJ, Bronson R, Crowley D, et al. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell. 2005;7(4):363–73.
100. Gressner O, Schilling T, Lorenz K, Schulze Schleithoff E, Koch A, Schulze-Bergkamen H, et al. TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. EMBO J. 2005;24(13):2458–71. PMCID: 1173149.
101. Guo X, Keyes WM, Papazoglu C, Zuber J, Li W, Lowe SW, et al. TAp63 induces senescence and suppresses tumorigenesis in vivo. Nat Cell Biol. 2009;11(12):1451–7. PMCID: 2920298.
102. Bourdon JC. p53 Family isoforms. Curr Pharm Biotechnol. 2007;8(6):332–6.
103. Chatterjee A, Chang X, Sen T, Ravi R, Bedi A, Sidransky D. Regulation of p53 family member isoform DeltaNp63alpha by the nuclear factor-kappaB targeting kinase IkappaB kinase beta. Cancer Res. 2010;70(4):1419–29. PMCID: 2963198.
104. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, et al. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell. 2009; 137(1):87–98.
105. Deyoung MP, Ellisen LW. p63 and p73 in human cancer: defining the network. Oncogene. 2007;26(36):5169–83.
106. Koster MI, Huntzinger KA, Roop DR. Epidermal differentiation: transgenic/knockout mouse models reveal genes involved in stem cell fate decisions and commitment to differentiation. J Investig Dermatol Symp Proc. 2002;7(1):41–5.
107. Burstein DE, Nagi C, Kohtz DS, Lee L, Wang B. Immunodetection of GLUT1, p63 and phospho-histone H1 in invasive head and neck squamous carcinoma: correlation of immunohistochemical staining patterns with keratinization. Histopathology. 2006;48(6):717–22.
108. Rocco JW, Leong CO, Kuperwasser N, DeYoung MP, Ellisen LW. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. Cancer Cell. 2006;9(1):45–56.
109. Saintigny P, El-Naggar AK, Papadimitrakopoulou V, Ren H, Fan YH, Feng L, et al. DeltaNp63 overexpression, alone and in combination with other biomarkers, predicts the development of oral cancer in patients with leukoplakia. Clin Cancer Res. 2009;15(19):6284–91. PMCID: 2756317.
110. Brooks CL, Gu W. p53 ubiquitination: Mdm2 and beyond. Mol Cell. 2006;21(3):307–15.
111. Marine JC, Francoz S, Maetens M, Wahl G, Toledo F, Lozano G. Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ. 2006;13(6): 927–34.

112. Zeng X, Chen L, Jost CA, Maya R, Keller D, Wang X, et al. MDM2 suppresses p73 function without promoting p73 degradation. Mol Cell Biol. 1999;19(5):3257–66. PMCID: 84120.
113. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, et al. NUMB controls p53 tumour suppressor activity. Nature. 2008;451(7174):76–80.
114. Abe Y, Oda-Sato E, Tobiume K, Kawauchi K, Taya Y, Okamoto K, et al. Hedgehog signaling overrides p53-mediated tumor suppression by activating Mdm2. Proc Natl Acad Sci U S A. 2008;105(12):4838–43. PMCID: 2290789.
115. Jung CR, Lim JH, Choi Y, Kim DG, Kang KJ, Noh SM, et al. Enigma negatively regulates p53 through MDM2 and promotes tumor cell survival in mice. J Clin Invest. 2010;120(12): 4493–506. PMCID: 2993588.
116. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev. 2003;13(1):77–83.
117. Song MS, Song SJ, Kim SY, Oh HJ, Lim DS. The tumour suppressor RASSF1A promotes MDM2 self-ubiquitination by disrupting the MDM2-DAXX-HAUSP complex. EMBO J. 2008;27(13):1863–74. PMCID: 2486425.
118. Kruse JP, Gu W. Modes of p53 regulation. Cell. 2009;137(4):609–22.
119. Li Y, Zhou Z, Chen C. WW domain-containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates apoptosis. Cell Death Differ. 2008;15(12):1941–51.
120. Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell. 2003;11(1):11–23.
121. Li Q, Lin S, Wang X, Lian G, Lu Z, Guo H, et al. Axin determines cell fate by controlling the p53 activation threshold after DNA damage. Nat Cell Biol. 2009;11(9):1128–34.
122. Bell HS, Dufes C, O'Prey J, Crighton D, Bergamaschi D, Lu X, et al. A p53-derived apoptotic peptide derepresses p73 to cause tumor regression in vivo. J Clin Invest. 2007;117(4):1008–18. PMCID: 1810568.
123. Sullivan A, Lu X. ASPP: a new family of oncogenes and tumour suppressor genes. Br J Cancer. 2007;96(2):196–200. PMCID: 2359998.
124. Jang H, Choi SY, Cho EJ, Youn HD. Cabin1 restrains p53 activity on chromatin. Nat Struct Mol Biol. 2009;16(9):910–5.
125. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C. Piccolo S. Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. Cell. 2003;113(3):301–14.
126. Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science. 2003;302(5652):1972–5.
127. Kanai M, Hanashiro K, Kim SH, Hanai S, Boulares AH, Miwa M, et al. Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly(ADP-ribosyl)ation. Nat Cell Biol. 2007;9(10):1175–83.
128. Marchenko ND, Wolff S, Erster S, Becker K, Moll UM. Monoubiquitylation promotes mitochondrial p53 translocation. EMBO J. 2007;26(4):923–34. PMCID: 1852828.
129. Mancini F, Di Conza G, Pellegrino M, Rinaldo C, Prodosmo A, Giglio S, et al. MDM4 (MDMX) localizes at the mitochondria and facilitates the p53-mediated intrinsic-apoptotic pathway. EMBO J. 2009;28(13):1926–39. PMCID: 2711189.
130. Sayan AE, Sayan BS, Gogvadze V, Dinsdale D, Nyman U, Hansen TM, et al. P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis. Oncogene. 2008;27(31):4363–72.
131. Morselli E, Tasdemir E, Maiuri MC, Galluzzi L, Kepp O, Criollo A, et al. Mutant p53 protein localized in the cytoplasm inhibits autophagy. Cell Cycle. 2008;7(19):3056–61.
132. Shi D, Pop MS, Kulikov R, Love IM, Kung AL, Grossman SR. CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proc Natl Acad Sci U S A. 2009;106(38):16275–80. PMCID: 2752525.
133. He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network-another piece in the tumour-suppression puzzle. Nat Rev Cancer. 2007;7(11):819–22.

134. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene. 2007;26(34):5017–22.
135. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26(5):745–52. PMCID: 1939978.
136. Kumar B, Cordell KG, Lee JS, Worden FP, Prince ME, Tran HH, et al. EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. J Clin Oncol. 2008;26(19):3128–37. PMCID: 2744895.
137. Ogawa T, Saiki Y, Shiga K, Chen N, Fukushige S, Sunamura M, et al. miR-34a is downregulated in cis-diamminedichloroplatinum treated sinonasal squamous cell carcinoma patients with poor prognosis. Cancer Sci. 2012;103(9):1737–43.
138. Guida E, Bisso A, Fenollar-Ferrer C, Napoli M, Anselmi C, Girardini JE, et al. Peptide aptamers targeting mutant p53 induce apoptosis in tumor cells. Cancer Res. 2008;68(16): 6550–8.
139. Alsner J, Jensen V, Kyndi M, Offersen BV, Vu P, Borresen-Dale AL, et al. A comparison between p53 accumulation determined by immunohistochemistry and *TP53* mutations as prognostic variables in tumours from breast cancer patients. Acta Oncol. 2008;47(4):600–7.
140. Hashimoto T, Tokuchi Y, Hayashi M, Kobayashi Y, Nishida K, Hayashi S, et al. p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. Cancer Res. 1999;59(21):5572–7.
141. Hofseth LJ, Saito S, Hussain SP, Espey MG, Miranda KM, Araki Y, et al. Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. Proc Natl Acad Sci U S A. 2003;100(1):143–8. PMCID: 140909.
142. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, et al. Tumour biology: senescence in premalignant tumours. Nature. 2005;436(7051):642.
143. Nenutil R, Smardova J, Pavlova S, Hanzelkova Z, Muller P, Fabian P, et al. Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. J Pathol. 2005;207(3):251–9.
144. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature. 2008;455(7216):1069–75. PMCID: 2694412.
145. Ahrendt SA, Halachmi S, Chow JT, Wu L, Halachmi N, Yang SC, et al. Rapid p53 sequence analysis in primary lung cancer using an oligonucleotide probe array. Proc Natl Acad Sci U S A. 1999;96(13):7382–7. PMCID: 22094.
146. Wikman FP, Lu ML, Thykjaer T, Olesen SH, Andersen LD, Cordon-Cardo C, et al. Evaluation of the performance of a p53 sequencing microarray chip using 140 previously sequenced bladder tumor samples. Clin Chem. 2000;46(10):1555–61.
147. Tennis M, Krishnan S, Bonner M, Ambrosone CB, Vena JE, Moysich K, et al. p53 Mutation analysis in breast tumors by a DNA microarray method. Cancer Epidemiol Biomarkers Prev. 2006;15(1):80–5.
148. Koch WM, Brennan JA, Zahurak M, Goodman SN, Westra WH, Schwab D, et al. p53 mutation and locoregional treatment failure in head and neck squamous cell carcinoma. J Natl Cancer Inst. 1996;88(21):1580–6.
149. Tandon S, Tudur-Smith C, Riley RD, Boyd MT, Jones TM. A systematic review of p53 as a prognostic factor of survival in squamous cell carcinoma of the four main anatomical subsites of the head and neck. Cancer Epidemiol Biomarkers Prev. 2010;19(2):574–87.
150. Sano D, Xie TX, Ow TJ, Zhao M, Pickering CR, Zhou G, et al. Disruptive *TP53* mutation is associated with aggressive disease characteristics in an orthotopic murine model of oral tongue cancer. Clin Cancer Res. 2011;17(21):6658–70. PMCID: 3207013.
151. Lindenbergh-van der Plas MB, Snijders PJ, et al. Prognostic significance of truncating *TP53* mutations in head and neck squamous cell carcinoma. Clin Cancer Res. 2011;17(11):3733–41.
152. Shoemaker RH. The NCI60 human tumour cell line anticancer drug screen. Nat Rev Cancer. 2006;6(10):813–23.

153. Cortinovis DL, Andriani F, Livio A, Fabbri A, Perrone F, Marcomini B, et al. FHIT and p53 status and response to platinum-based treatment in advanced non-small cell lung cancer. Curr Cancer Drug Targets. 2008;8(5):342–8.
154. Mandic R, Schamberger CJ, Muller JF, Geyer M, Zhu L, Carey TE, et al. Reduced cisplatin sensitivity of head and neck squamous cell carcinoma cell lines correlates with mutations affecting the COOH-terminal nuclear localization signal of p53. Clin Cancer Res. 2005;11(19 Pt 1):6845–52.
155. Bradford CR, Zhu S, Ogawa H, Ogawa T, Ubell M, Narayan A, et al. P53 mutation correlates with cisplatin sensitivity in head and neck squamous cell carcinoma lines. Head Neck. 2003;25(8):654–61.
156. Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet. 2003;33(3):357–65.
157. Gudkov AV, Komarova EA. The role of p53 in determining sensitivity to radiotherapy. Nat Rev Cancer. 2003;3(2):117–29.
158. van Houten VM, Leemans CR, Kummer JA, Dijkstra J, Kuik DJ, van den Brekel MW, et al. Molecular diagnosis of surgical margins and local recurrence in head and neck cancer patients: a prospective study. Clin Cancer Res. 2004;10(11):3614–20.
159. Harden SV, Thomas DC, Benoit N, Minhas K, Westra WH, Califano JA, et al. Real-time gap ligase chain reaction: a rapid semiquantitative assay for detecting p53 mutation at low levels in surgical margins and lymph nodes from resected lung and head and neck tumors. Clin Cancer Res. 2004;10(7):2379–85.
160. Poeta ML, Manola J, Goldenberg D, Forastiere A, Califano JA, Ridge JA, et al. The Ligamp *TP53* Assay for Detection of Minimal Residual Disease in Head and Neck Squamous Cell Carcinoma Surgical Margins. Clin Cancer Res. 2009;15(24):7658–65. PMCID: 2842212.
161. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6(5):963–8.
162. Partridge M, Pateromichelakis S, Phillips E, Emilion GG, A'Hern RP, Langdon JD. A case-control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization. Cancer Res. 2000;60(14):3893–8.
163. el-Naggar AK, Lai S, Luna MA, Zhou XD, Weber RS, Goepfert H, et al. Sequential p53 mutation analysis of pre-invasive and invasive head and neck squamous carcinoma. Int J Cancer. 1995;64(3):196–201.
164. Koch WM, Boyle JO, Mao L, Hakim J, Hruban RH, Sidransky D. p53 gene mutations as markers of tumor spread in synchronous oral cancers. Arch Otolaryngol Head Neck Surg. 1994;120(9):943–7.
165. Pateromichelakis S, Farahani M, Phillips E, Partridge M. Molecular analysis of paired tumours: time to start treating the field. Oral Oncol. 2005;41(9):916–26.
166. Garcia-Cao I, Garcia-Cao M, Martin-Caballero J, Criado LM, Klatt P, Flores JM, et al. "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. EMBO J. 2002;21(22):6225–35. PMCID: 137187.
167. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. Cell. 2006;127(7):1323–34.
168. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression in vivo. Nature. 2007;445(7128):661–5.
169. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature. 2007;445(7128):656–60.
170. Bischoff JR, Kirn DH, Williams A, Heise C, Horn S, Muna M, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science. 1996;274(5286):373–6.
171. Khuri FR, Nemunaitis J, Ganly I, Arseneau J, Tannock IF, Romel L, et al. a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nat Med. 2000;6(8):879–85.

172. Nemunaitis J, Khuri F, Ganly I, Arseneau J, Posner M, Vokes E, et al. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. J Clin Oncol. 2001;19(2):289–98.
173. Zeimet AG, Marth C. Why did p53 gene therapy fail in ovarian cancer? Lancet Oncol. 2003;4(7):415–22.
174. Ulasov IV, Tyler MA, Han Y, Glasgow JN, Lesniak MS. Novel recombinant adenoviral vector that targets the interleukin-13 receptor alpha2 chain permits effective gene transfer to malignant glioma. Hum Gene Ther. 2007;18(2):118–29.
175. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):711–23.
176. Chiappori AA, Soliman H, Janssen WE, Antonia SJ, Gabrilovich DI. INGN-225: a dendritic cell-based p53 vaccine (Ad.p53-DC) in small cell lung cancer: observed association between immune response and enhanced chemotherapy effect. Expert Opin Biol Ther. 2010;10(6): 983–91. PMCID: 3146364.
177. Selivanova G, Iotsova V, Okan I, Fritsche M, Strom M, Groner B, et al. Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. Nat Med. 1997;3(6):632–8.
178. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Pharmacological rescue of mutant p53 conformation and function. Science. 1999;286(5449):2507–10.
179. Cheok CF, Verma CS, Baselga J, Lane DP. Translating p53 into the clinic. Nat Rev Clin Oncol. 2011;8(1):25–37.
180. Xie X, Piao L, Bullock BN, Smith A, Su T, Zhang M, et al. Targeting HPV16 E6-p300 interaction reactivates p53 and inhibits the tumorigenicity of HPV-positive head and neck squamous cell carcinoma. Oncogene. 2013. doi: 10.1038/onc.
181. Kojima K, Konopleva M, Sumudio IJ, Shikami M, Cabriera-Hansen M, McQueen T, Ruvolo V, Tsao T, Zeng Z, Vassilev LT, Andreef M. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. Blood. 2005;106(9):3150–9.
182. Carter BZ, Mak DH, Schober WD, Koller E, Pinilla C, Vassilev LT, Reed JC, Andreef M. Simultaneous activation of p53 and inhibition of XIAP enhance the activation of apoptosis signaling pathways in AML. Blood. 2010;115(2):306–14.
183. Shinohara ET, Subhawong TK, Geng L, Kim KW, Albert JM, Hallahan DE, Lu B. Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2. Mol Cancer Ther. 2006;5(2):411–7.
184. Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med. 2004;10(12):1321–8.
185. Enge M, Bao W, Hedstrom E, Jackson SP, Moumen A, Selivanova G. MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. Cancer Cell. 2009;15(3):171–83.
186. Roh JL, Ko JH, Moon SJ, Ryu CH, Choi JY, Koch WM. The p53-reactivating small-molecule RITA enhances cisplatin-induced cytotoxicity and apoptosis in head and neck cancer. Cancer Lett. 2012;325(1):35–41.
187. Terzian T, Suh YA, Iwakuma T, Post SM, Neumann M, Lang GA, et al. The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes Dev. 2008;22(10):1337–44. PMCID: 2377188.
188. Luu Y, Bush J, Cheung KJ Jr, Li G. The p53 stabilizing compound CP-31398 induces apoptosis by activating the intrinsic Bax/mitochondrial/caspase-9 pathway. Exp Cell Res. 2002;276(2):214–22.
189. Tang X, Zhu Y, Han L, Kim AL, Kopelovich L, Bickers DR, et al. CP-31398 restores mutant p53 tumor suppressor function and inhibits UVB-induced skin carcinogenesis in mice. J Clin Invest. 2007;117(12):3753–64. PMCID: 2096455.
190. Rao CV, Swamy MV, Patlolla JM, Kopelovich L. Suppression of familial adenomatous polyposis by CP-31398, a *TP53* modulator, in APCmin/+ mice. Cancer Res. 2008;68(18):7670–5. PMCID: 2597360.

191. Chipuk JE, Maurer U, Green DR, Schuler M. Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer Cell. 2003;4(5):371–81.
192. Weinmann L, Wischhusen J, Demma MJ, Naumann U, Roth P, Dasmahapatra B, et al. A novel p53 rescue compound induces p53-dependent growth arrest and sensitises glioma cells to Apo2 L/TRAIL-induced apoptosis. Cell Death Differ. 2008;15(4):718–29.
193. Roh JL, Kang SK, Minn I, Califano JA, Sidransky D, Koch WM. p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. Oral Oncol. 2011;47(1):8–15. PMCID: 3032831.

Chapter 13
DNA Damage Proteins and Response to Therapy in Head and Neck Cancer

Ranee Mehra and Ilya G. Serebriiskii

Abstract DNA-damaging agents such as cisplatin and radiotherapy have provided a cornerstone of treatment for squamous cell cancers of the head and neck (SCCHN). Cells possess multiple mechanisms for repairing different classes of DNA damage, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), resolution of intrastrand cross-linking by the Fanconi anemia (FA) proteins, nonhomologous end joining (NHEJ), and homologous recombination (HR). Preclinical and a limited degree of clinical research has focused on evaluating whether changes in expression, mutation, or polymorphic variants in the many enzymes involved in these DNA repair pathways are involved in treatment resistance in SCCHN. This chapter will first summarize the proteins functioning in the complementary DNA-damage response pathways, then focus on the current data regarding their prognostic value in the clinic, noting the limitations of current retrospective evaluations, and discussing implications for future research.

Keywords DNA repair · Cisplatin adducts · Radiation

13.1 Introduction

Squamous cell carcinomas of the head and neck (SCCHN) have a multitude of somatic genetic alterations, many of which impact the ability of the cells to repair damaged DNA. This has prompted investigators to study polymorphisms in DNA-repair genes in order to determine if there is any increase in the susceptibility of an

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individual to develop SCCHN [1–5]. From a therapeutic perspective, there has also been interest in pathways related to treatment efficacy. The cornerstone of nonsurgical treatment for locally advanced SCCHN has been a combination of cytotoxic chemotherapy, biological targeted agents, and radiation to work in concert to damage cellular DNA, thus initiating a cascade of events that result in apoptosis [6–9]. While there are many factors associated with drug sensitivity, molecular aberrations in DNA repair may, in part, make tumor cells more sensitive to DNA-damaging agents [10]. Reciprocally, resistance to therapy likely stems in many cases from the enhanced abilities of tumor cells to repair DNA damage. A greater understanding of how the DNA repair machinery regulates treatment efficacy and resistance in primary SCCHN is gradually emerging, although progress has been hampered by the complexity of DNA repair systems and the lack of reliable clinical assays for biomarkers of DNA damage repair.

Cisplatin, an integral component of SCCHN treatment both in the curative and palliative settings, exerts its cytotoxic effects via the formation of platinum-DNA adducts [11–15]. The presence of adducts triggers cell-cycle arrest and DNA repair mechanisms: If repair is unsuccessful, cells progress to aberrant mitosis and eventual apoptosis [16]. Similarly, ionizing radiation often induces its cytotoxic effects by inducing double-strand breaks (DSBs). While the combination of cisplatin and radiation can cure a high percentage of locally advanced SCCHN, there is still a significant proportion of patients, especially among those with human papillomavirus (HPV)-negative SCCHN, with tumors that are intrinsically resistant to treatment, who develop recurrent disease [17], [18]. Thus, understanding the role of DNA repair in treatment resistance, in particular among the poor prognosis HPV-negative patients, is essential to the development of better therapies. This chapter will focus on a review of relevant DNA-repair pathways in the treatment of SCCHN, current data regarding DNA repair proteins and outcomes in SCCHN, and future directions for the clinical development of treatments based on observed protein expression and function of a group of DNA repair proteins.

13.2 DNA Repair Overview

DNA repair pathways are complex and involve numerous enzymes working in tandem to recognize, remove, repair, and resynthesize damaged DNA. Common pathways and their functions, listed in Table 13.1, include base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR); and in the case of DSBs, nonhomologous end joining (NHEJ) and homologous recombination (HR), and the repair of DNA cross-linking mediated by the Fanconi anemia (FA) system. Cisplatin and radiation, the most common interventions in the treatment of SCCHN, typically are repaired through action of the NER and DSB machinery [7]. Recent thorough mechanistic reviews of the DNA repair machinery for each pathway are cited in the text below for reference: Our focus here is on introducing those under investigation in SCCHN.

Table 13.1 Key enzymatic pathways in DNA repair

Pathway	Function	Repair of specified lesion	Key enzymes involved
Excision Repair			
Base excision repair (BER)	Removal of damaged base followed by synthesis and ligation	Single-strand breaks	XRCC1 PARP DNA glycosylase APEX1 POLβ PNKP DNA ligase
Nucleotide excision repair (NER)	Removal of damaged region (multiple nucleotides) followed by repair	DNA adduct removal	ERCC1 XPF (ERCC4) XPC-RAD23B CSA (ERCC8) CSB (ERCC6) XPD (ERCC2) XPB (ERCC3) XPA XPG (ERCC5)
Mismatch repair (MMR)	Repair of incorrect base pairing		MSH2 MLH1 DNA polymerase MSH6 PMS2
Repair of strand breaks			
Nonhomologous end joining (NHEJ)	Bind exposed ends of a DNA break, even without complementary nucleotides	Double-strand breaks	Ku70 Ku80 DNA-PK PNKP XLF XRCC4/LIG4
Homologous recombination (HR)	Repair of broken DNA ends	Double-strand breaks	BRCA1 BRCA2 RAD50 RAD51 RAD52 ATM MRE11 ATR
Fanconi anemia pathway		DNA interstrand cross-links	FANCA, B, C, E, F, G, I, L, M FANCD2 FANCD1(BRCA2) FANCJ(PALB2) FANCO(RAD51)

Because of the fundamental role of many of these enzymes in protecting cells from UV damage and other forms of damage, mutations in NER and HR genes have been classically associated with genetic syndromes, resulting in potential confusion in nomenclature. Hence, while the formal nomenclature for genes discussed below

include designations as part of the X-ray repair cross-complementation (XRCC) and excision-repair cross-complementation (ERCC) groups, these genes are also named based on their involvement in Cockayne's syndrome (CS) or xeroderma pigmentosum (XP). All of the proteins noted below are potential targets of biomarker studies. The short summary below illustrates the complexity of the interactions and redundancy among the various DNA repair pathways.

Base Excision Repair (BER) BER (Fig. 13.1) replaces nucleotides that have been damaged by alkylating agents, radiation, oxidation, or deamination, with the first two defects particularly linked to SCCHN therapies. Kim and Wilson have written an excellent recent review of this mechanism [19]. BER focuses on the removal of nonbulky adducts; bulky adducts are instead processed through the NER process described below. In BER, damaged bases are first recognized by a DNA glycosylase, which recognizes and removes an incorrect base, creating an abasic site. Second, the abasic site is recognized by an apurinic/apyrimidinic (AP) endonuclease, of which APEX1 is the most commonly active in humans. Third, a group of proteins including APEX1 but also DNA polymerase β (POLβ) and polynucleotide kinase 3′-phosphatase (PNKP) "clean" the termini of the excised residue, rendering the site suitable for final repair by elimination of inappropriate chemical groups. Fourth, a DNA polymerase complex including POLβ fills the remaining gap in the DNA with the appropriate base; depending on the length of the lesion, either long patch (LP) or short patch (SP)-BER occurs, with involvement of slightly different protein complexes. Fifth, the nick is sealed by DNA ligase III (LIG3) in a complex with X-ray repair cross-complementing group 1 (XRCC1), a nonenzymatic scaffolding protein that supports its activity [20].

In single-strand break (SSB) repair, the nick-sensing activity of poly(ADP-ribose) polymerase (PARP) enzymes is also important. In the PARP family of enzymes, the most common subtype is PARP1 [21], [22], which consists of an NH2-terminal DNA-damage recognition domain, a modification domain, and a catalytic domain. PARP1 has been a central target of anticancer therapies, in part due to its function in the detection of SSB [23]. There is considerable interest in synergy between PARP inhibition and the HR pathway (discussed below), especially in tumors that are deficient in key enzymes in HR, including BRCA1 and BRCA2 [24], [25]. This synergy stems from the inability of HR-deficient cells to repair replication-associated DSBs that result from persistent SSB's [26].

Nucleotide Excision Repair (NER) Given the widespread use of cisplatin in the treatment of SCCHN, it is not surprising that mechanisms to repair platinum-induced DNA damage have been a topic of much study. Cisplatin exerts its cytotoxic effects via the formation of platinum-DNA adducts. NER (Fig. 13.2), which typically repairs ultraviolet (UV)-induced DNA adduct formation, is also a key process in the repair of cisplatin induced damage [27–29]. There are two main subtypes of NER, the global genomic NER (GG-NER) pathway, and the transcription-coupled NER (TC-NER) pathway. While the GG-NER pathway uses specific recognition proteins that scan the genome to identify any sites of DNA damage, the TC-NER uses a different recognition mechanism for those regions of the genome that are transcriptionally active. Both converge on downstream factors that mediate DNA repair: Overall, the

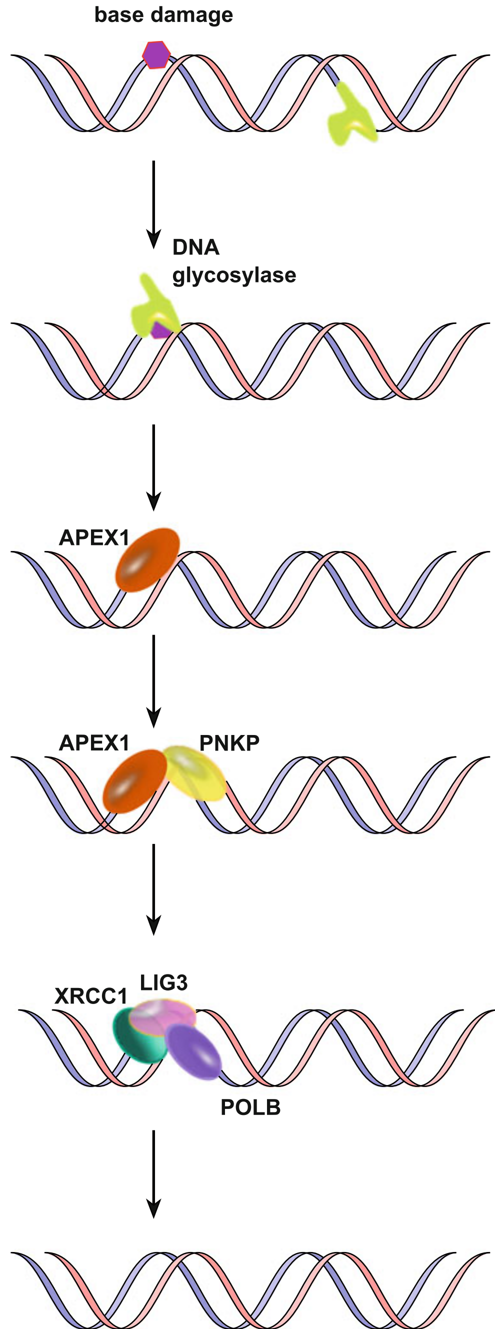

Fig. 13.1 Representation of the base excision repair (BER) pathway. In some cases where genes have an official gene symbol and a common name, the official symbol is used, i.e., APEX1/APE1 (see text for details)

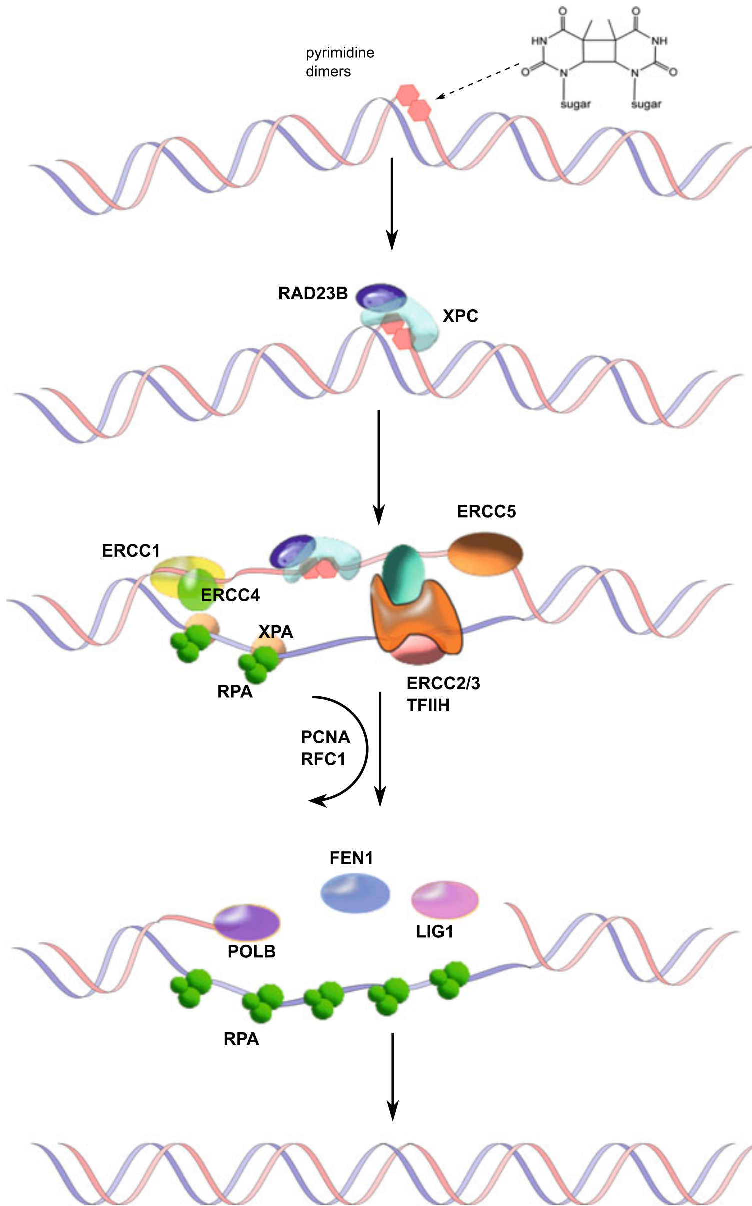

Fig. 13.2 Representation of the nucleotide excision repair (NER) pathway. In some cases where genes have an official gene symbol and a common name, the official symbol is used: ERCC2/XPD, ERCC3/XPB, ERCC4/XPF, ERCC5/XPG, RFC1/RFC

NER pathway comprises at least 23 enzymes, including the ERCC1-XPF(ERCC4) complex. Detailed mechanistic reviews of GG-NER and TC-NER have recently appeared in a review by Iyama et al., while http://repairtoire.genesilico.pl/ provides a useful searchable database [30].

In GG-NER, the XPC-RAD23B complex recognizes DNA breaks. These proteins bind DNA on the opposite strand from the abnormal adduct. In TC-NER, in areas of active transcription, RNA polymerase encounters a defective base, stalls, and recruits two additional proteins, CSA(ERCC8) and CSB(ERCC6). For each form of NER, these recognition complexes then recruit a multiprotein repair assembly including the TFIIH transcription-associated complex, which contains the helicases XPD(ERCC2) and XPB(ERCC3), which unwind the DNA in the region of DNA damage. Additional proteins XPA and replication protein A (RPA) are recruited to this unwound region, and form a local preincision complex in which XPA binds and recruits the ERCC1-XPF(ERCC4) heterodimer and XPG(ERCC5). The ERCC1-XPF(ERCC4) dimer recognizes the damaged DNA and cuts 5′ to the lesion; this is often a critical rate-limiting step in the repair process. The catalytic domain of the nuclease is located on XPF, and ERCC1 is responsible for DNA binding and stabilization [31–33]. In parallel, XPG cleaves 3′ to the lesion. Subsequently, the defective base is removed, and binding of proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) allow DNA polymerases to load to the site, and subsequently fill in the gap. Final closure of the lesion is mediated by XRCC1-LIG3, as in BER, or an alternative ligation complex involving FEN1-LIG1.

ERCC1 and XPF(ERCC4) have attracted considerable study in SCCHN, particularly as potential biomarkers of effective NER activity relevant to DNA-damaging therapies. ERCC1 knockout mice are often not viable and die from liver failure, and in humans *ERCC1* gene mutations result in severe deformities with cerebrooculofacioskeletal syndrome, implying critical function [34]. Preclinical studies in lung cancer and Chinese hamster ovary (CHO) cell lines have shown that ERCC1(-) lines which lack the capacity for UV damage repair were more sensitive to the cytotoxic effects of cisplatin than those transfected with the human ERCC1 gene [28]. In addition, ERCC1(-) cells did not repair any DNA adduct lesions in a 24-h period, in contrast to 48 % repair in ERCC1 (+) cells. When the same experiments were conducted in XPB(ERCC3)-positive or -negative cells, there was no variance in platinum sensitivity or DNA repair, indicating that ERCC3 is likely not as critical for platinum sensitivity.

The ERCC1-XPF(ERCC4) complex also has a role in the repair of DSBs, often resulting from ionizing radiation [35]. Although DSBs typically are repaired by either HR or nonhomologous end joining (NHEJ), it has been shown that murine and human cells deficient in ERCC1-XPF are sensitive to irradiation with a resulting increase in γH2AX foci, a marker of DSB injury. The role of ERCC1/XPF(ERCC4) in DSB differs from HR and NHEJ, and requires further study [35].

Mismatch Repair (MMR) MMR is an editing process for newly synthesized DNA, highly conserved throughout evolution [36]. Dominant negative mutations in human genes involved in MMR, including MLH1, MSH2, MSH6, and PMS2, result in microsatellite instability and are associated with hereditary cancers such as Lynch syndrome (hereditary nonpolyposis colorectal cancer). Mutations in these genes have also been implicated in other sporadic cancers. To date, relatively little is known about the relevance of MMR in SCCHN.

Repair of Double-Strand Breaks (DSBs): Homologous Recombination (HR) and Nonhomologous End Joining (NHEJ) Both the HR (Fig. 13.3) and NHEJ (Fig. 13.4) pathways are important for the repair of DSBs [16], [30], [37]. With HR, an intact DNA strand is usually present, repair is often error free, and typically occurs in the S and G2 phases in the cell cycle. In contrast, NHEJ can happen at any point in the cell cycle, but can result in further DNA damage, including the initiation of translocation events [38].

In HR, initial sensing of the break is mediated by three proteins (MRE11, RAD50, and Nijmegen breakage syndrome 1 (NBS1)) known collectively as the MRN complex. The MRN complex recruits and activates the ataxia telangiectasia mutated (ATM) kinase, triggering a cell cycle checkpoint. Further, ATM-dependent phosphorylation of histone 2A H2AX, family member X, results in the formation of the γ-H2AX complex, which in turn leads to recruitment of other repair enzymes, including BRCA1 [39–41]. MRN also recruits C-terminal binding protein-interacting protein (CtIP), which (along with other exonucleases, e.g., EXO1) trims the ends of the break to generate single strand overhangs. Single-stranded regions are protected and stabilized by being coated with RPA, which also recruits BRCA2, RAD51, and RAD52, resulting in formation of Rad51-single strand DNA (ssDNA) nucleoprotein filaments. RPA also assists in recruiting and activating a second checkpoint kinase, ATR, strengthening cell-cycle arrest. Single-stranded DNA bound by RAD51 is capable of invading a homologous template, and initiating Holliday junction-mediated repair.

In the NHEJ repair system, initial recognition of the break is made by the Ku78/Ku80 heterodimer, which binds to the DNA ends directly. These proteins then recruit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and together this trimeric complex aligns the two broken ends [42], [43]. End processing enzymes (e.g., Artemis) then generate terminal overhangs suitable for ligation, followed by PNKP to ensure the proper phosphorylation. Finally, XLF and the XRCC4/LIG4 complex together close the gap.

The Fanconi Anemia (FA) pathway in Head and Neck Cancer FA, an autosomal recessive disorder, is a genetically heterogeneous disease that is associated with an array of phenotypes including bone marrow failure, birth defects, and hypersensitivity to DNA interstrand cross-link (ICL) damage [44], [45]. Interestingly, patients with FA are at greater risk of developing head and neck cancer [46], [47]. An intact FA pathway likely suppresses HPV infection, consistent with emerging data that FA syndrome patients also have an increased risk of susceptibility to HPV induced oncogenesis [48–50]. Mutations in approximately 15 genes have been linked to FA, including FANCA, B, C, D2, E, F G, I, L, and M; in addition, mutations in BRCA2(FANCD1), BRIP1(FANCJ), and PALB2(FANCJ), RAD51C(FANCO), and SLX4(FANCP) can result in FA phenotypes [44], [45]. Many of these FANC proteins act as a complex triggering "unhooking" of intrastrand cross-links introduced by platinum-based chemotherapies or radiation, with the cleavage activity catalyzed by ERCC1-XPF(ERCC4) [51], [52], and also MUS81–EME1, SLX1 and FAN1 [53], [54]. Fanconi gene mutations have been associated with reduced platinum resistance, with mutations of FANCC and FANCD2 genes in SCCHN cell lines inducing more sensitivity to cisplatin [55].

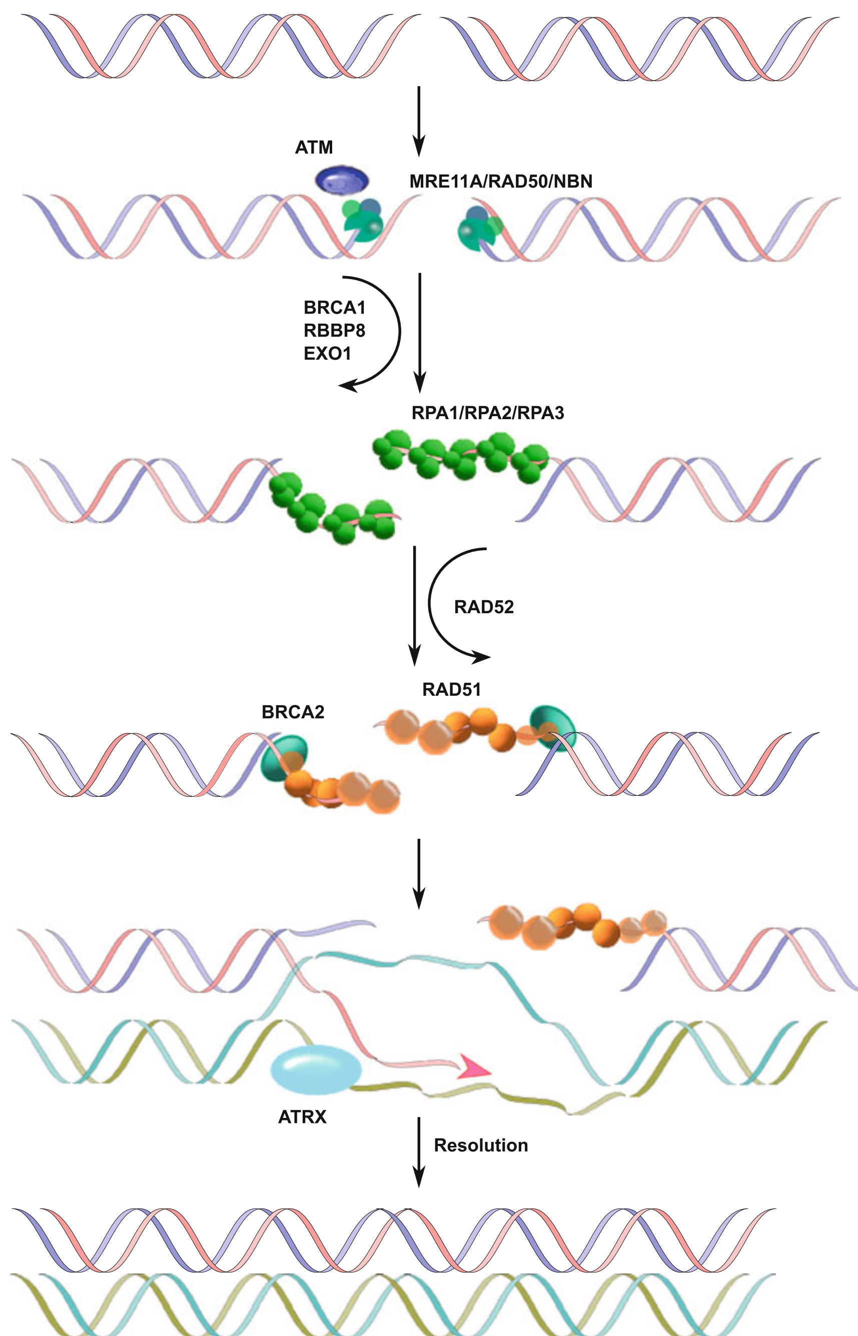

Fig. 13.3 Representation of the homologous recombination (HR) pathway. In some cases where genes have an official gene symbol and a common name, the official symbol is used: NBN/NBS1, H2AFX/H2AX, RBBP8/CTIP (see text for details)

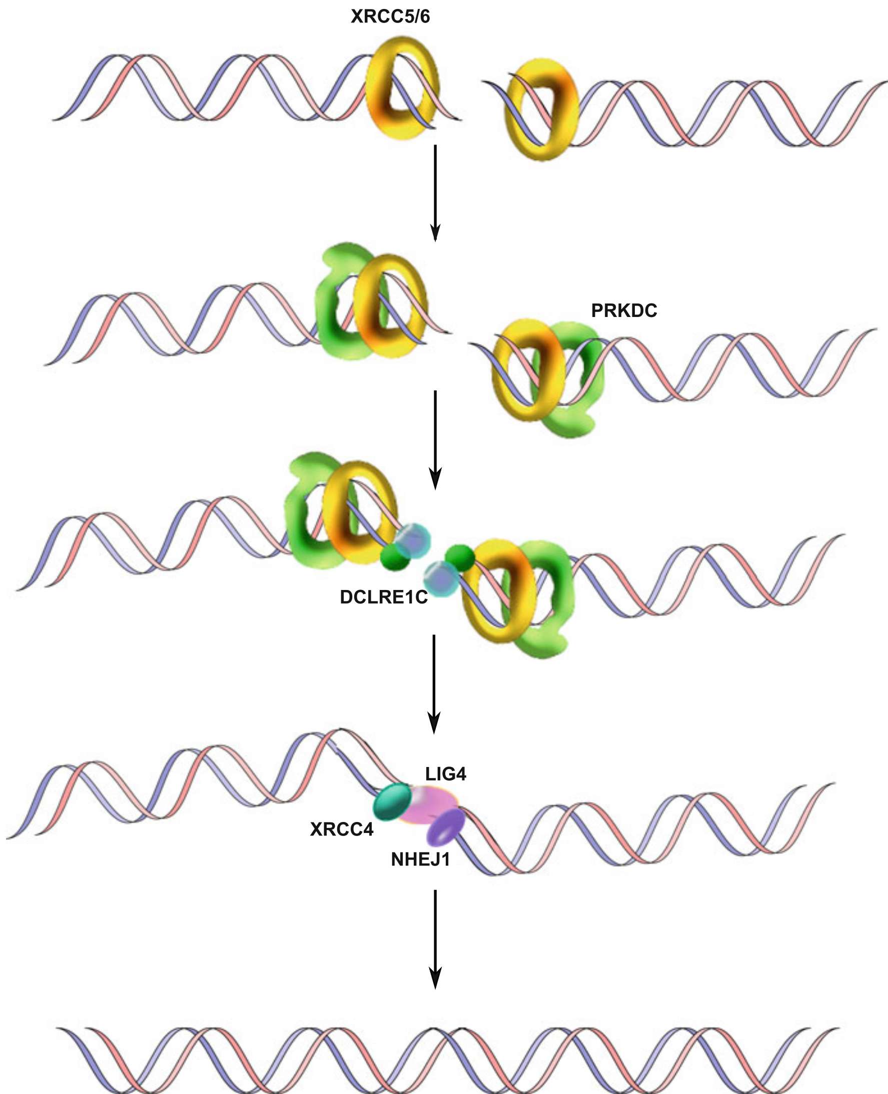

Fig. 13.4 Representation of the nonhomologous end joining (NHEJ) pathway. In some cases where genes have an official gene symbol and a common name, the official symbol is used: XRCC5/Ku80, XRCC6/Ku70, NHEJ1/XLF, PRKDC/DNA-PKcs, DCLRE1C/ARTEMIS (see text for details)

13.3 Use of DNA Repair Proteins as Biomarkers of Therapeutic Response

A major focus in SCCHN and in other cancers has been to exploit DNA repair proteins as biomarkers for likelihood of response to DNA damaging therapies. Challenges to the routine use of DNA repair biomarkers have been hampered by the effective

development of reliable, validated assays. One example, involving antibodies to visualize ERCC1 using an immunohistochemistry (IHC) approach, is discussed in some detail below to emphasize the difficulty with this approach. Other approaches for measurement of critical enzymes in each of these pathways have included RT-PCR-based assays and an analysis of polymorphisms. Given the concerns regarding biomarker validation, most of the biomarkers of DNA repair described in this chapter are being studied in the research realm, but are not yet used in routine clinical practice. Many proteins shown to function in DNA repair have not yet been analyzed at all as biomarkers for response in SCCHN.

13.3.1 Nucleotide Excision Repair Biomarkers: ERCC1 and XPF(ERCC4)

ERCC1 Biomarkers in the NER pathway have been a subject of extensive study in SCCHN, in part due to the widespread use of cisplatin and radiation therapy. Interest in ERCC1 as a biomarker began in non-small cell lung cancer, where small retrospective studies showed a longer survival among patients who received platinum-based chemotherapy and had low ERCC1 tumor levels, as measured by RT-PCR, [56]. This finding was reinforced by a larger effort to study ERCC1 expression in tumor samples from the International Adjuvant Lung Trial, which indicated that patients with ERCC1-negative tumors had a greater overall survival with the addition of adjuvant chemotherapy [57]. A pathologist-generated H-score, which factors the percentage and intensity of positive cells, was determined for 761 tumors and correlated with survival. The results indicated that patients with ERCC1-negative tumors had a greater survival benefit with the addition of adjuvant chemotherapy. Reaction to these results was guarded, however, since there were substantial concerns regarding the specificity of the antibody which was used, 8F1 (Lab Vision) [58]. Years after the original publication, the authors of the IALT-Bio study published a report questioning the operating characteristics of later lots of 8F1, given their inability to reproduce their original results [59]. Their report also called into question the importance of ERCC1 isoforms; of four characterized isoforms, only ERCC1-202 has been shown to be functionally active, as this was the only isoform able to reinstate platinum resistance in ERCC-deficient cell lines. Currently, all known ERCC1 antibodies detect several isoforms and thus it is not possible to only detect ERCC1-202 with immunohistochemistry.

In the study of SCCHN, many small retrospective analyses also utilized the ERCC1 antibody 8F1 [60–64]. These have shown mixed results with regards to the association of ERCC1 expression and survival, possibly due to the variability of this antibody, and the mixed population of HPV (+) and (−) disease. One retrospective series, published by Handra-Luca et al., analyzed 96 SCCHN tumor specimens for ERCC1 IHC with 8F1, quantifying staining with an H-score, which factors the percentage and intensity of positive cells [63]. All patients in this analysis underwent primary treatment with induction cisplatin and 5-fluorouracil followed by radiation therapy and 71 % of tumors had high expression levels. The results were intriguing,

with an odds ratio of response of 4.3 in the ERCC1 low group with the multivariate analysis, and a corresponding greater survival in this group (40.5 months) compared to the ERCC1 high group (27.7 months). ERCC1 low expression was associated with a reduced risk of cancer-related death (HR 0.42).

Given the need for more reliable antibodies to assay for ERCC1, recent publications have focused on additional reagents. One highly specific anti-ERCC1 antibody, FL-297 (Santa Cruz), [65] is hampered by variable nonspecific cytoplasmic staining in formalin fixed paraffin-embedded tissue, dampening enthusiasm for its use as an IHC reagent. FL-297 has been used to study clinical specimens for ERCC1 expression, also with mixed results [66]. Additional reagents under study include 4F9 (Origene) and HPA029773 (Sigma), both of which have been shown to be specific for ERCC1 [66–68].

Bauman and colleagues presented their analysis of tissue from a randomized phase II study of cisplatin and radiation therapy with or without erlotinib for patients with locally advanced SCCHN [67], [68]. Tumors were quantified for ERCC1 H-score using recent batches of 8F1, FL297, and 4F9. 8F1 revealed no association between ERCC1 expression and response rate (RR), progression-free survival (PFS), or overall survival (OS). In contrast, higher expression with either FL297 or 4F9 was associated with a shorter PFS (HR 3.55 and 3.54, respectively).

Hao et al. reviewed ERCC1 expression in a cohort of 55 tumor samples from patients with locally advanced SCCHN treated with weekly cisplatin with radiation [69]. 8F1 and FL-297 were used and ERCC1 was measured with a quantitative fluorescent immunohistochemistry platform (AQUA, HistoRx) [70], [71]. This technology differs from traditional IHC methodology in that data appear as a continuous output of AQUA scores, precise quantification of fluorescent signaling allows for reproducible and quantitative results, and permits subcellular localization of the antigen signal of interest, which is especially important for a target such as ERCC1, which should have mainly nuclear activity. For both antibodies, AQUA scores were dichotomized into high and low expressing groups, with only 22 % agreement between the two reagents. Their results showed no correlation between response and ERCC1 expression with either antibody. ERCC1 expression measured by FL297, but not 8F1, was associated with a 4.4-fold increase ($p = .004$) in overall survival when adjusted for age, primary site, smoking history, T/N stage and HPV status.

At Fox Chase Cancer Center, a retrospective analysis was also conducted on tissue from resected SCCHN [66]. Treatment in this series was mainly primary surgery, with high-risk patients receiving adjuvant treatment with radiation or platinum and radiation. Three antibodies were used, including 8F1 and FL297, discussed above, and HPA029773 (Sigma). Using a 2009 lot of 8F1 (Lab Vision), and FL297, a tissue microarray from 105 HPV-negative patients was stained and analyzed with AQUA. Cut points to determine ERCC1 expression level (high vs. low) were determined with the Classification and Regression Tree (CART) methodology. Using the "old" 8F1, there was a statistically significant improvement in median survival among patients who received surgery plus adjuvant radiation and had low ERCC1 expressing tumors as compared to ERCC1 high; $p = 0.036$, HR 2.35. However, while there was a trend towards a greater survival in the same group of patients when tumor ERCC1

levels were assayed with FL297, this was not statistically significant ($p = 0.19$), and considerable cytoplasmic staining with FL297 was noted. For the analysis involving HPA029773, data from additional tumors were added to the previous set, with similar patient and treatment characteristics to increase the number of patients who received surgery plus adjuvant radiation/chemoradiation ($n = 80$). Again, after adjusting for patient age, gender, and tumor/nodal stage, an overall median survival of 64 months in the low ERCC1 group and 29 months in the high ERCC1 group was observed among patients who were treated with adjuvant therapy ($p = 0.02$, HR 2.72).

The majority of analyses of ERCC1 in SCCHN have focused on IHC-based assays, rather than measuring mRNA levels with quantitative RT-PCR (reverse transcriptase polymerase chain reaction). The latter has been utilized in the study of non-small cell lung, esophageal, and ovarian cancers, although with variable results [56], [72–76]. It is important to note that RT-PCR-based assays have not been validated in prospective randomized trials, and it is not known if this approach is optimal for assaying DNA repair functional capacity [77]. For instance, a measure of message levels, rather than protein expression does not account for differing rates of genes transcription, posttranslational modification, and protein stability. In addition, accurate and reproducible results are greatly dependent on the quality of the extracted mRNA, which may be more variable in archival specimens [78]. The limited data on ERCC1 mRNA expression in SCCHN do not support an association between mRNA levels and survival or response rate to platinum chemotherapy [69].

XPF(ERCC4) This catalytic component of the ERCC1-XPF complex has been studied less frequently as a potential biomarker of DNA repair in SCCHN. Vaezi et al. evaluated the feasibility of XPF immunodetection using a specific and well-characterized antibody (SPM228, Abcam) [79]. XPF expression was variable in tissue from a heterogeneous group of 80 patients, but overall there was a higher expression in tumor as compared to paired normal tissue. Among the patients assessed, most had locally advanced disease, with either the oropharynx or oral cavity as primary sites. These patients had received a variety of treatment approaches, including definitive chemoradiation or surgery followed by adjuvant therapy. In aggregate, all patients received radiation therapy and 88 % received platinum-based therapy. Both H-score and a quantitative readout of XPF expression were employed with good agreement (Spearman rank correlation = 0.78, $p < 0.001$). Oral cavity primary sites, which would not be expected to be associated with HPV, had high expression levels of XPF. With regard to clinical outcome, in multivariate analysis, there was a statistically significant association between high XPF expression and early recurrence ($p = 0.01$). XPF protein expression has also been quantified in a cohort of 13 SCCHN cell lines derived from a variety of primary sites, and correlated with cisplatin sensitivity [80]. Compared to testis cell lines, SCCHN cells had higher levels of XPF expression, which was associated with increased cisplatin resistance in those in vitro models.

13.3.2 Base Excision Repair Biomarker: XRCC1

Ang and colleagues analyzed tissue by IHC from 138 SCCHN patients who were treated with a variety of modalities including surgery, surgery followed by adjuvant treatment, and definitive chemoradiation [81]. Among the patients treated with chemoradiation, high expression of XRCC1 was associated with poorer progression-free and overall survival, with this effect primarily observed in patients who had received chemoradiation: no correlation of XRCC1 and survival was seen in patients who had received surgery. This survival effect was independent of the survival effect associated with p16 expression status.

13.3.3 Double-Strand Repair Biomarkers: RAD51, Ku70, Ku80

Radiation damage is opposed by repair of DSBs, with increased repair capacity associated with radiation failure [82]. RAD51, a key component of the HR pathway, was evaluated in a small retrospective series of 12 patients who were treated with induction chemotherapy followed by chemoradiation [83]. Even with this limited sample size, there was a significant difference in cancer-specific survival based on RAD51 expression, with high expression levels being associated with worse outcomes (33 % vs. 89 % at 2 years, $p = 0.025$). In a study of the rate of error-prone NHEJ repair, SCCHN cells had less error-free repair compared to normal human oral keratinocytes [84]. In a retrospective analysis of 75 SCCHN tumors from patients treated with a combination of chemotherapy and radiation, higher Ku70 mRNA levels were associated with a greater response to treatment and longer recurrence free survival [85]. This result was somewhat unexpected, and in contrast, in a study of Ku80 expression, high Ku80 protein levels were associated with increased risk of local failure and mortality ($p = 0.01$). This association was strongest among the HPV-negative subgroup [86].

13.4 Single Nucleotide Polymorphisms (SNPs) as Predictors of Treatment Resistance

Single nucleotide polymorphisms involving ERCC1 and XPF have been studied as potential biomarkers of cisplatin resistance. Thirteen single nucleotide allelic variants were studied in association with PFS in a cohort of patients with head and neck cancer [79]. Four SNPs (rs3136155(CT/TT; HR = 2.0, raw $p = 0.053$), rs1799799 (TC/CC; HR = 1.94, raw $p = 0.065$), rs3136202 (GA/AA; HR = 1.94, raw $p = 0.065$), and rs31336166 (TG/GG; HR = 1.94, raw $p = 0.065$)) were marginally associated with PFS. However, these SNPs were located in introns and were not shown to impact XPF expression levels. Thus, it is unclear how relevant the presence of these SNPs may be with respect to XPF function and treatment

resistance. Hao et al., in the same group of 55 tumors analyzed by IHC to assess ERCC1 expression, looked at ERCC1 genotypes in 41 patients (C/C, C/T, and T/T genotype frequencies were 17.1 %, 43.9 %, and 39.0 %) and found no difference in response rate between C/C, C/T, and T/T genotypes [69]. In contrast, other investigators looked at tumors from 103 patients and used polymerase chain reaction-restriction fragment length polymorphism (RFLP) to look for SNPs in XPD-Asp312Asn, XPD-Lys751Gln, ERCC1-Cys809Ala, and XRCC1-Arg399Gln [87]. The median overall survival was not reached for patients who had at least one polymorphic variant ($p < .001$). Increasing numbers of variants resulted in increasing protection from death and increased chances of a complete response ($p = 0.041$).

Additional polymorphisms which have been shown to be associated with improved outcomes in SCCHN patients treated with chemoradiation include ERCC2 A35931C, but these results are preliminary and based on one retrospective series [88]. Another focus of research has included the association of DNA repair polymorphisms, such as XRCC1 c.1196A > G and RAD51 c.-3429 G > C, and an increased risk of acute toxicities related to chemoradiation [89]. Given the morbidity of chemoradiation, the ability to predict which patients may be at increased risk could eventually have potential clinical applications.

13.5 The Cancer Genome Atlas (TCGA): Profiles of DDR Proteins in SCCHN

As of late 2013, information on 295 SCCHN tumors was made publicly available based on work of the TCGA Consortium. Figure 13.5 represents data on mRNA expression, copy number, and mutation for genes functioning in the NER, BER, HR, and NHEJ pathways. Elevated expression or an increased copy number is the most common change for the majority of these genes, most consistently reported for genes associated with BER. Typically, these changes are observed in no more than 15–20 % of tumors for any given gene. An interesting and plausible possibility is that for genes operating in a common pathway, changes in function in any one of a group of functionally interacting group of proteins will increase pathway activity. In this case, a predictor of resistance to radiation or cisplatin might be a distributed diagnostic signature, e.g., hypothetically, elevation of any one of the genes in the group ERCC1-ERCC5 would produce some resistance, particularly if coupled with elevation in expression of PCNA or RAD23B.

Deletions among this group of genes appear to be rare, with the highest frequency observed for ATM at 2 %. However, a subset of genes is mutated in 2–6 % of SCCHN tumors, including ATM, ATRX, BRCA1, BRCA2, and PRKDC. Mutations in some of these genes have been associated with pathogenesis of other types of cancer [90–92]. The consequences of these mutations have not been extensively investigated in SCCHN, but may well predict specific prognosis or therapeutic response. The potential to exploit these genomic resources in trials of strategies to better stratify patients to receive high versus low doses of DNA-damaging therapies is apparent.

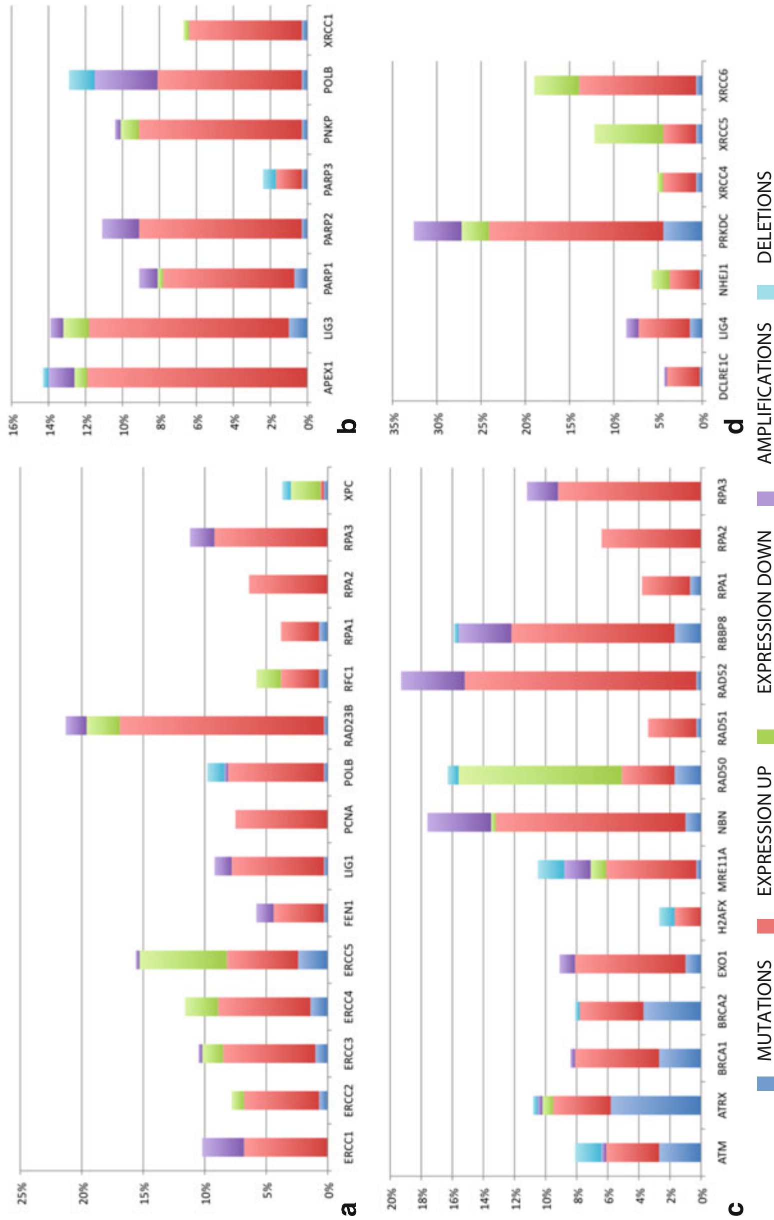

Fig. 13.5 TCGA analysis of the **a** NER, **b** BER, **c** HR, and **d** NHEJ pathways. For each of the genes belonging to the DNA repair pathways depicted in Figs. 13.1–13.4, information on expression levels, copy number variations and mutations was extracted from the provisional TCGA data set for head and neck squamous cell carcinoma (295 completely analyzed and annotated tumors). (Data were extracted from the http://www.cbioportal.org/. The cutoff for expression was set as z-score = +/−2. Note: Some of the proteins indicated function in more than one pathway; see text for details)

13.6 DNA Repair and HPV in SCCHN

In all of these reports regarding the impact of ERCC1 expression on survival among patients who are treated with either radiation or chemotherapy, there appears to be a signal that this is especially of interest in the HPV-negative population. For instance, the Fox Chase analysis, which showed an association between ERCC1 and survival, was conducted exclusively in an HPV-negative population. In addition, the work published by Hao and colleagues indicates that patients who were HPV-negative with high ERCC1 tumor levels had a worse prognosis than HPV-positive patients. Martins et al. showed that p16-negative tumors were more likely to have normal to high ERCC1 levels. Similarly, Vaezi et al. also noted increased XPF levels in oral cavity tumors, which are usually not HPV related. Finally, in the series reported on by Moeller and colleagues, the association between Ku80 expression and survival was strongest in the HPV-negative population [86]. Thus, these retrospective reports illustrate the importance of integrating HPV status into analysis of DNA damage-related biomarkers; hopefully future prospective studies will study the potential relationship with HPV and DNA repair biomarkers further.

13.7 Cancer Signaling, DNA Repair, and Therapeutic Targets

The epidermal growth factor receptor (EGFR) is expressed in a majority of SCCHN and is an established therapeutic target [11], [12], [93–95]. Preclinical studies have indicated that its activity in part may be related to modulation of DNA repair. In particular, the EGFR inhibitor cetuximab is a standard radiation sensitizer for locally advanced, potentially curable SCCHN. In vitro data from SCCHN cells lines indicate that cetuximab strongly inhibits postradiation DNA damage repair and results in redistribution of DNA-PK out of the nucleus [17]. Similarly, the small molecule EGFR inhibitor gefitinib reduces DNA-PK levels in SCCHN cell lines [96]. In addition, in a different model of prostate cancer cell lines, the combination of EGF ligand and radiation resulted in upregulation of XRCC1 and ERCC1, presumably resulting in increased DNA repair capacity. This modulation of XRCC1 and ERCC1 seemed to occur via MAPK signaling and was attenuated by MEK inhibition [97].

One recent study has identified another potentially important mechanism for regulation of ERCC1 expression. Epithelial-mesenchymal transition (EMT) plays a significant role in cancer metastasis, and there is increasing evidence that resistance to therapy also is associated with EMT [98–100]. Snail1, a transcription factor, is related to EMT and metastasis in SCCHN [101]. Hsu et al. have extensively evaluated the relationship between Snail1 and ERCC1 in SCCHN cell lines and tissues, and how this relates to cisplatin resistance [102]. In cell lines, increased levels of Snail expression were associated with high ERCC1 expression as measured by the polyclonal antibody FL297, which recognizes ERCC1. Similarly, suppression of Snail expression resulted in low levels of ERCC1 and diminished resistance to cisplatin. In 72 SCCHN tumors, co-overexpression of Snail and ERCC1 was noted ($p = 0.002$)

and associated with an inferior survival among patients who were treated with cisplatin. The authors showed that Snail directly regulates ERCC1 transcription, and that platinum resistance is dependent on the presence of ERCC1, results suggesting a multitude of avenues for further study.

As a greater understanding of DNA repair pathways and the impact of treatment develops, efficacy can be optimized with newer biologically targeting agents. For instance, the Hsp90 inhibitor NVP-AUY922 may impair RAD51 mediated double DSB repair, thus allowing for further radiosensitization [103]. Similarly, the VEGFR/PDGFR/RAF inhibitor sorafenib has been shown to downregulate ERCC1 and XRCC1 levels in a dose-dependent fashion among in vitro studies with chemoradiation combinations [104]. While the PARP inhibitor ABT-888 has been of considerable interest in BRCA-mutated breast cancers, preclinical studies have suggested potential synergy between this agent and radiation in head and neck cancer cell lines, likely due to attenuation of NHEJ repair and decreased DNA repair [105]. Thus, there is considerable potential for incorporation of DNA repair modulating agents in future clinical studies.

13.8 Future Considerations and Conclusions

Incorporation of components of DNA repair pathways as integral biomarkers in therapeutic clinical trials for SCCHN is currently limited. This is in part due to the lack of available clinically validated assays. It remains to be seen whether either evaluation of protein expression or of message levels would be the optimal predictive marker for sensitivity of chemotherapy of radiation. As future studies interrogate these markers further, it will be important that assays are reliable, reproducible, and validated appropriately before they are incorporated into clinical development.

As illustrated by this chapter, due to the complexities of DNA repair pathways, investigation in this area is fraught with controversies and questions. Trends from retrospective analyses of human cancer tissue seem to indicate that higher expression and thus potential activity of DNA repair enzymes may be associated with an inferior response to treatment and survival. Questions that arise include the impact of HPV-induced pathogenesis on the activity of DNA repair pathways. Future research will need to focus on optimization of biomarker assays that can be used to reliably study patient samples. Eventually, prospective clinical trials will be essential in order to validate biomarker studies and therapeutic interventions.

References

1. Sturgis EM, Clayman GL, Guan Y, Guo Z, Wei Q. DNA repair in lymphoblastoid cell lines from patients with head and neck cancer. Arch Otolaryngol Head Neck Surg. 1999 Feb;125(2):185–90.
2. Sturgis EM, Zheng R, Li L, et al. XPD/ERCC2 polymorphisms and risk of head and neck cancer: a case-control analysis. Carcinogenesis. 2000 Dec;21(12):2219–23.

3. Sturgis EM, Castillo EJ, Li L, et al. XPD/ERCC2 EXON 8 Polymorphisms: rarity and lack of significance in risk of squamous cell carcinoma of the head and neck. Oral Oncol. 2002 Jul;38(5):475–77.
4. Jones NR, Spratt TE, Berg AS, Muscat JE, Lazarus P, Gallagher CJ. Association studies of excision repair cross-complementation group 1 (ERCC1) haplotypes with lung and head and neck cancer risk in a Caucasian population. Cancer Epidemiol. 2011 Apr;35(2):175–81.
5. Jelonek K, Gdowicz-Klosok A, Pietrowska M, et al. Association between single-nucleotide polymorphisms of selected genes involved in the response to DNA damage and risk of colon, head and neck, and breast cancers in a Polish population. J Appl Genet. 2010;51(3):343–52.
6. Chinnaiyan P, Huang S, Vallabhaneni G, et al. Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). Cancer Res. 15 Apr;2005;65(8):3328–35.
7. Sak A, Grehl S, Engelhard M, et al. Long-term in vivo effects of cisplatin on gamma-H2AX foci signaling in peripheral lymphocytes of tumor patients after irradiation. Clin Cancer Res. Apr 15 2009;15(8):2927–34.
8. Bonner JA, Raisch KP, Trummell HQ, et al. Enhanced apoptosis with combination C225/radiation treatment serves as the impetus for clinical investigation in head and neck cancers. J Clin Oncol. 2000 Nov 1;18(21 Suppl):47S–53S.
9. Harari PM, Huang SM. Head and neck cancer as a clinical model for molecular targeting of therapy: combining EGFR blockade with radiation. Int J Radiat Oncol Biol Phys. 2001 Feb 1;49(2):427–33.
10. Rusin P, Walczak A, Zwierzchlejska A, et al. DNA damage and repair of head and neck cancer cells after radio- and chemotherapy. Zeitschrift fur Naturforschung. 2009 Jul-Aug;64(7-8):601–10.
11. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008 Sep 11;359(11):1116–27.
12. Burtness B, Goldwasser MA, Flood W, Mattar B, Forastiere AA. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005 Dec 1;23(34):8646–54.
13. Adelstein DJ, Li Y, Adams GL, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. J Clin Oncol. 2003 Jan 1;21(1):92–8.
14. Blommaert FA, Floot BG, van Dijk-Knijnenburg HC, et al. The formation and repair of cisplatin-DNA adducts in wild-type and cisplatin-resistant L1210 cells: comparison of immunocytochemical determination with detection in isolated DNA. Chem Biol Interact. 1998 Jan 30;108(3):209–25.
15. Forastiere AA, Zhang Q, Weber RS, et al. Long-term results of RTOG 91–11: a comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol. 2013 Mar 1;31(7):845–52.
16. Cha HJ, Yim H. The accumulation of DNA repair defects is the molecular origin of carcinogenesis. Tumour Biol. 2013 Aug 2.
17. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. Clin Cancer Res. 2000 Jun;6(6):2166–74.
18. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008 Feb 20;100(4):261–69.
19. Kim YJ, Wilson DM 3rd. Overview of base excision repair biochemistry. Curr Mol Pharmacol. 2012 Jan;5(1):3–13.
20. Vidal AE, Boiteux S, Hickson ID, Radicella JP. XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions. EMBO J. 2001 Nov 15;20(22):6530–39.

21. Chambon P, Weill JD, Mandel P. Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. Biochem Biophys Res Commun. 1963 Apr 2;11:39–43.
22. Uchida K, Miwa M. Poly(ADP-ribose) Polymerase: structural conservation among different classes of animals and its implications. Mol Cell Biochem. 1994 Sep;138(1-2):25–32.
23. Fisher AE, Hochegger H, Takeda S, Caldecott KW. Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase. Mol Cell Biol. 2007 Aug;27(15):5597–605.
24. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005 Apr 14;434(7035):917–21.
25. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005 Apr 14;434(7035):913–17.
26. Javle M, Curtin NJ. The role of PARP in DNA repair and its therapeutic exploitation. Br J Cancer. 2011 Oct 11;105(8):1114–22.
27. Zamble DB, Mu D, Reardon JT, Sancar A, Lippard SJ. Repair of cisplatin-DNA adducts by the mammalian excision nuclease. Biochem. 1996 Aug 635(31):10004–13.
28. Lee KB, Parker RJ, Bohr V, Cornelison T, Reed E. Cisplatin sensitivity/resistance in UV repair-deficient Chinese hamster ovary cells of complementation groups 1 and 3. Carcinog. 1993 Oct;14(10):2177–80.
29. Houtsmuller AB, Rademakers S, Nigg AL, Hoogstraten D, Hoeijmakers JH, Vermeulen W. Action of DNA repair endonuclease ERCC1/XPF in living cells. Science. 1999 May 7;284(5416):958–61.
30. Iyama T, Wilson DM 3rd. DNA repair mechanisms in dividing and non-dividing cells. DNA Repair. 2013 Aug;12(8):620–36.
31. Enzlin JH, Scharer OD. The active site of the DNA repair endonuclease XPF-ERCC1 forms a highly conserved nuclease motif. EMBO J. 2002 Apr 15;21(8):2045–53.
32. Tsodikov OV, Enzlin JH, Scharer OD, Ellenberger T. Crystal structure and DNA binding functions of ERCC1, a subunit of the DNA structure-specific endonuclease XPF-ERCC1. Proc Natl Acad Sci U S A. 2005 Aug 9;102(32):11236–41.
33. Bessho T, Sancar A, Thompson LH, Thelen MP. Reconstitution of human excision nuclease with recombinant XPF-ERCC1 complex. J Biol Chem. 1997 Feb 7;272(6):3833–37.
34. Jaspers NG, Raams A, Silengo MC, et al. First reported patient with human ERCC1 deficiency has cerebro-oculo-facio-skeletal syndrome with a mild defect in nucleotide excision repair and severe developmental failure. Am J Hum Genet. 2007 Mar;80(3):457–66.
35. Ahmad A, Robinson AR, Duensing A, et al. ERCC1-XPF endonuclease facilitates DNA double-strand break repair. Mol Cell Biol. 2008 Aug;28(16):5082–92.
36. Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. Mech Ageing Dev. 2008 Jul-Aug;129(7-8):391–407.
37. Misteli T, Soutoglou E. The emerging role of nuclear architecture in DNA repair and genome maintenance. Nat Rev Mol Cell Biol. 2009 Apr;10(4):243–54.
38. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol. 2003 Aug;23(16):5706–15.
39. Fernandez-Capetillo O, Celeste A, Nussenzweig A. Focusing on foci: H2AX and the recruitment of DNA-damage response factors. Cell Cycle. 2003 Sep-Oct;2(5):426–27.
40. Lee SA, Baker MD. Analysis of DNA repair and recombination responses in mouse cells depleted for Brca2 by SiRNA. DNA Repair. 2007 Jun 1;6(6):809–17.
41. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem. 2004;73:39–85.
42. Gottlieb TM, Jackson SP. The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell. 1993 Jan 15;72(1):131–42.
43. Yang J, Yu Y, Hamrick HE, Duerksen-Hughes PJ. ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. Carcinogenesis. 2003 Oct;24(10):1571–80.
44. Grompe M, D' Andrea A. Fanconi anemia and DNA repair. Hum Mol Genet. 2001 Oct 1;10(20):2253–59.

45. Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature. 2013 Jan 17;493(7432):356–63.
46. Kutler DI, Auerbach AD, Satagopan J, et al. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg. 2003 Jan;129(1):106–12.
47. Kutler DI, Singh B, Satagopan J, et al. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood. 2003 Feb 15;101(4):1249–56.
48. Hoskins EE, Morreale RJ, Werner SP, et al. The fanconi anemia pathway limits human papillomavirus replication. J Virol. 2012 Aug;86(15):8131–38.
49. Kutler DI, Wreesmann VB, Goberdhan A, et al. Human papillomavirus DNA and p53 polymorphisms in squamous cell carcinomas from Fanconi anemia patients. J Natl Cancer Inst. 2003 Nov 19;95(22):1718–21.
50. Lowy DR, Gillison ML. A new link between Fanconi anemia and human papillomavirus-associated malignancies. J Natl Cancer Inst. 2003 Nov 19;95(22):1648–50.
51. Orelli B, McClendon TB, Tsodikov OV, Ellenberger T, Niedernhofer LJ, Scharer OD. The XPA-binding domain of ERCC1 is required for nucleotide excision repair but not other DNA repair pathways. J Biol Chem. 2009 Feb 5;285(6):3705–12.
52. Murray D, Rosenberg E. The importance of the ERCC1/ERCC4[XPF] complex for hypoxic-cell radioresistance does not appear to derive from its participation in the nucleotide excision repair pathway. Mutat Res. 1996 Dec 2;364(3):217–26.
53. Bhagwat N, Olsen AL, Wang AT, et al. XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. Mol Cell Biol. 2009 Dec;29(24):6427–37.
54. Thyagarajan B, Campbell C. Elevated homologous recombination activity in fanconi anemia fibroblasts. J Biol Chem. 1997 Sep 12;272(37):23328–33.
55. Kachnic LA, Li L, Fournier L, Willers H. Fanconi anemia pathway heterogeneity revealed by cisplatin and oxaliplatin treatments. Cancer Lett. 2010 Jun 1;292(1):73–79.
56. Lord RV, Brabender J, Gandara D, et al. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. Clin Cancer Res. 2002 Jul;8(7):2286–91.
57. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med. 2006 Sep 7;355(10):983–91.
58. Niedernhofer LJ, Bhagwat N, Wood RD. ERCC1 and non-small-cell lung cancer. N Engl J Med. 2007 Jun 14;356(24):2538-2540; author reply 2540–1.
59. Friboulet L, Olaussen KA, Pignon JP, et al. ERCC1 isoform expression and DNA repair in non-small-cell lung cancer. N Engl J Med. 2013 Mar 21;368(12):1101–10.
60. Jun HJ, Ahn MJ, Kim HS, et al. ERCC1 expression as a predictive marker of squamous cell carcinoma of the head and neck treated with cisplatin-based concurrent chemoradiation. Br J Cancer. 2008 Jul 8;99(1):167–72.
61. Fountzilas G, Bamias A, Kalogera-Fountzila A, et al. Induction chemotherapy with docetaxel and cisplatin followed by concomitant chemoradiotherapy in patients with inoperable non-nasopharyngeal carcinoma of the head and neck. Anticancer Res. 2009 Feb;29(2):529–38.
62. Seiwert TY, Cohen EE, Wang X, et al. Use of systematic analysis of DNA repair pathways in head and neck cancer (HNC) to identify XPF as a novel predictor of induction response, and pMK2 relationship to chemoradiotherapy. J Clin Oncol. 2008;26(15S): 6003.
63. Handra-Luca A, Hernandez J, Mountzios G, et al. Excision repair cross complementation group 1 immunohistochemical expression predicts objective response and cancer-specific survival in patients treated by Cisplatin-based induction chemotherapy for locally advanced head and neck squamous cell carcinoma. Clin Cancer Res. 2007 Jul 1;13(13):3855–59.
64. Koh Y, Kim TM, Jeon YK, et al. Class III beta-tubulin, but not ERCC1, is a strong predictive and prognostic marker in locally advanced head and neck squamous cell carcinoma. Ann Oncol. 2009 Aug;20(8):1414–19.
65. Bhagwat NR, Roginskaya VY, Acquafondata MB, Dhir R, Wood RD, Niedernhofer LJ. Immunodetection of DNA repair endonuclease ERCC1-XPF in human tissue. Cancer Res. 2009 Sep 1;69(17):6831–38.

66. Mehra R, Zhu F, Yang DH, et al. Quantification of excision repair cross complementing group 1 (ERCC1) and survival in p16-negative squamous cell head and neck cancers. Clin Cancer Res. 2013 Oct 2.
67. Austin M, Schmidt R, Parvathaneni U, et al. Expression of p16, ERCC1, and EGFR amplification as predictors of responsiveness of locally advanced squamous cell carcinomas of head and neck (SCCHN) to cisplatin, radiotherapy, and erlotinib: A phase II randomized trial. Paper presented at: ASCO2012.
68. Bauman JE, Austin MC, Schmidt R, et al. ERCC1 is a prognostic biomarker in locally advanced head and neck cancer: results from a randomised, phase II trial. Br J Cancer. 2013 Oct 15;109(8):2096–105.
69. Hao D, Lau HY, Eliasziw M, et al. Comparing ERCC1 protein expression, mRNA levels, and genotype in squamous cell carcinomas of the head and neck treated with concurrent chemoradiation stratified by HPV status. Head Neck. 2011 Jun;34(6):785–91.
70. Gustavson MD, Bourke-Martin B, Reilly DM, et al. Development of an unsupervised pixel-based clustering algorithm for compartmentalization of immunohistochemical expression using automated quantitative analysis. Appl Immunohistochem Mol Morphol. 2009 Jul;17(4):329–37.
71. Moeder CB, Giltnane JM, Moulis SP, Rimm DL. Quantitative, fluorescence-based in-situ assessment of protein expression. Methods Mol Biol. 2009;520:163–75.
72. Ceppi P, Volante M, Novello S, et al. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. Ann Oncol. 2006 Dec;17(12):1818–25.
73. Cobo M, Isla D, Massuti B, et al. Customizing cisplatin based on quantitative excision repair cross-complementing 1 mRNA expression: a phase III trial in non-small-cell lung cancer. J Clin Oncol. 2007 Jul 1;25(19):2747–54.
74. Joshi MB, Shirota Y, Danenberg KD, et al. High gene expression of TS1, GSTP1, and ERCC1 are risk factors for survival in patients treated with trimodality therapy for esophageal cancer. Clin Cancer Res. 2005 Mar 15;11(6):2215–21.
75. Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. J Clin Invest. 1994 Aug;94(2):703–8.
76. Simon G, Sharma A, Li X, et al. Feasibility and efficacy of molecular analysis-directed individualized therapy in advanced non-small-cell lung cancer. J Clin Oncol. 2007 Jul 1;25(19):2741–46.
77. Bepler G, Williams C, Schell MJ, et al. Randomized international phase III trial of ERCC1 and RRM1 expression-based chemotherapy versus gemcitabine/carboplatin in advanced non-small-cell lung cancer. J Clin Oncol. 2013 Jul 1;31(19):2404–12.
78. Vermeulen J, De Preter K, Lefever S, et al. Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res. 2011 May;39(9):e63.
79. Vaezi A, Wang X, Buch S, et al. XPF expression correlates with clinical outcome in squamous cell carcinoma of the head and neck. Clin Cancer Res. 2011 Aug 15;17(16):5513–22.
80. Koberle B, Ditz C, Kausch I, Wollenberg B, Ferris RL, Albers AE. Metastases of squamous cell carcinoma of the head and neck show increased levels of nucleotide excision repair protein XPF in vivo that correlate with increased chemoresistance ex vivo. Int J Oncol. 2010 May;36(5):1277–84.
81. Ang MK, Patel MR, Yin XY, et al. High XRCC1 protein expression is associated with poorer survival in patients with head and neck squamous cell carcinoma. Clin Cancer Res. 2011 Oct 15;17(20):6542–52.
82. Schwartz JL, Mustafi R, Beckett MA, Weichselbaum RR. DNA double-strand break rejoining rates, inherent radiation sensitivity and human tumour response to radiotherapy. Br J Cancer. 1996 Jul;74(1):37–42.
83. Connell PP, Jayathilaka K, Haraf DJ, Weichselbaum RR, Vokes EE, Lingen MW. Pilot study examining tumor expression of RAD51 and clinical outcomes in human head cancers. Int J Oncol. 2006 May;28(5):1113–19.

84. Shin KH, Kang MK, Kim RH, Kameta A, Baluda MA, Park NH. Abnormal DNA end-joining activity in human head and neck cancer. Int J Mol Med. 2006 May;17(5):917–24.
85. Pavon MA, Parreno M, Leon X, et al. Ku70 predicts response and primary tumor recurrence after therapy in locally advanced head and neck cancer. Int J Cancer. 2008 Sep 1;123(5): 1068–79.
86. Moeller BJ, Yordy JS, Williams MD, et al. DNA repair biomarker profiling of head and neck cancer: Ku80 expression predicts locoregional failure and death following radiotherapy. Clin Cancer Res. 2011 Apr 1;17(7):2035–43.
87. Quintela-Fandino M, Hitt R, Medina PP, et al. DNA-repair gene polymorphisms predict favorable clinical outcome among patients with advanced squamous cell carcinoma of the head and neck treated with cisplatin-based induction chemotherapy. J Clin Oncol. 2006 Sep 10;24(26):4333–39.
88. Zhong S, Nukui T, Buch S, et al. Effects of ERCC2 Lys751Gln (A35931C) and CCND1 (G870A) polymorphism on outcome of advanced-stage squamous cell carcinoma of the head and neck are treatment dependent. Cancer Epidemiol Biomarkers Prev. 2011 Nov;20(11):2429–37.
89. Pratesi N, Mangoni M, Mancini I, et al. Association between single nucleotide polymorphisms in the XRCC1 and RAD51 genes and clinical radiosensitivity in head and neck cancer. Radiother Oncol. 2011 Jun;99(3):356–61.
90. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. Nature Genet. 2013 Mar;45(3):279–84.
91. Kannan K, Inagaki A, Silber J, et al. Whole-exome sequencing identifies ATRX mutation as a key molecular determinant in lower-grade glioma. Oncotarget. Oct 2012;3(10):1194–1203.
92. de Miranda NF, Peng R, Georgiou K, et al. DNA repair genes are selectively mutated in diffuse large B cell lymphomas. J Exp Med. 2013 Aug 26;210(9):1729–42.
93. Sheikh Ali MA, Gunduz M, Nagatsuka H, et al. Expression and mutation analysis of epidermal growth factor receptor in head and neck squamous cell carcinoma. Cancer Sci. 2008 Aug;99(8):1589–94.
94. Baselga J, Trigo JM, Bourhis J, et al. Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck. J Clin Oncol. 2005 Aug 20;23(24):5568–77.
95. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006 Feb 9;354(6):567–78.
96. Magne N, Fischel JL, Tiffon C, et al. Molecular mechanisms underlying the interaction between ZD1839 ('Iressa') and cisplatin/5-fluorouracil. Br J Cancer. 2003 Aug 4;89(3): 585–92.
97. Yacoub A, McKinstry R, Hinman D, Chung T, Dent P, Hagan MP. Epidermal growth factor and ionizing radiation up-regulate the DNA repair genes XRCC1 and ERCC1 in DU145 and LNCaP prostate carcinoma through MAPK signaling. Radiat Res. 2003 Apr;159(4):439–52.
98. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002 Jun;2(6):442–54.
99. Yang AD, Fan F, Camp ER, et al. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. Clin Cancer Res. 2006 Jul 15; 12(14 Pt 1):4147–53.
100. Arumugam T, Ramachandran V, Fournier KF, et al. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. Cancer Res. 2009 Jul 15;69(14):5820–28.
101. Yang MH, Chang SY, Chiou SH, et al. Overexpression of NBS1 induces epithelial-mesenchymal transition and co-expression of NBS1 and Snail predicts metastasis of head and neck cancer. Oncog. 2007 Mar 1;26(10):1459–67.
102. Hsu DS, Lan HY, Huang CH, et al. Regulation of excision repair cross-complementation group 1 by Snail contributes to cisplatin resistance in head and neck cancer. Clin Cancer Res. 2010 Sep 15;16(18):4561–71.

103. Zaidi S, McLaughlin M, Bhide SA, et al. The HSP90 inhibitor NVP-AUY922 radiosensitizes by abrogation of homologous recombination resulting in mitotic entry with unresolved DNA damage. PloS One. 2012;7(4):e35436.
104. Yadav A, Kumar B, Teknos TN, Kumar P. Sorafenib enhances the antitumor effects of chemoradiation treatment by downregulating ERCC-1 and XRCC-1 DNA repair proteins. Mol Cancer Ther. 2011 Jul;10(7):1241–51.
105. Nowsheen S, Bonner JA, Yang ES. The poly(ADP-Ribose) polymerase inhibitor ABT-888 reduces radiation-induced nuclear EGFR and augments head and neck tumor response to radiotherapy. Radiother Oncol. 2011 Jun;99(3):331–8.

Chapter 14
Hypoxia and Radioresistance in Head and Neck Cancer

Peiwen Kuo and Quynh-Thu Le

Abstract Despite advances in radiotherapy, disease control in locally advanced (LA) head and neck squamous cell carcinoma (HNSCC) has seen marginal improvement. Hypoxia, a common occurrence in HNSCC, is associated with poor prognosis through protection of cells from radiation-induced DNA damage and alteration of tumor biology to promote a malignant progression. Significant effort has been devoted to targeting hypoxia in radiotherapy, including modification of tumor oxygenation, modification of the tumor vasculature, manipulation of tumor oxygen consumption and developing agents to either sensitize hypoxic cancer cells to radiation or to destroy them altogether. However, these approaches have had limited success in the clinic. Subsequent analyses of these studies revealed the importance of appropriate patient selection for hypoxia-targeted therapies. For example, it is now known that patients with human papilloma virus (HPV)-associated oropharyngeal carcinoma (OPC) do not benefit from hypoxia-targeted therapy. The future of hypoxia-targeted treatment in HNSCC radiotherapy lies in establishing a reliable and reproducible biomarker(s) or an imaging approach that can reflect tumor oxygenation and serve to select patients with aggressive tumors for therapy intensification.

Keywords Head and neck · Squamous cell carcinoma · Hypoxia · Radiation · Radioresistance · Oxygen enhancement ratio · Nitroimidazole · Tirapazamine · HPV · Pimonidazole · Fluoromisonidazole · Fluorodeoxyglucose · Fluoroazomycinarabinofuranoside · EF5 · HIF-1α

14.1 Introduction

Head and neck squamous cell carcinoma (HNSCC), including squamous cell carcinoma (SCC) of the oral cavity, oropharynx, hypopharynx and larynx, is one of the most lethal cancers worldwide. As the sixth most common cancer, with approximately 500,000 new cases each year, about 40 % of patients present with locally

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advanced disease (stages III and IV) [1]. For patients with advanced disese, median survival is around 10 months and 5-year survival does not exceed 40 % [2]. Among patients with distant metastases, the overall survival rate at 5 years is as low as 6.5 % [3]. Ultimately, many of these patients succumb to their disease because of inability to achieve locoregional control with standard therapy. Interestingly, in recent decades there has been a rapid rise in human papillomavirus (HPV)-related HNSCC, most commonly associated with HPV-16 in the oropharynx (OP). Considered clinically distinct from HPV-negative HNSCC, patients with HPV-positive OP tumors typically respond better to radiotherapy and chemotherapy, with an excellent overall survival exceeding 80 % [4].

Over the years, new radiation delivery techniques including intensity-modulated radiotherapy (IMRT) and image-guided radiotherapy (IGRT) have been used to deliver higher doses to targeted tumor volumes while sparing surrounding normal tissue, especially the parotid glands. Manipulation of radiation dose and schedule to reduce the overall time of the radiation course (accelerated fractionation) or to increase the total dose (hyperfractionation) have also been applied to address treatment failure from rapid tumor repopulation [5]. Despite improvement in tumor staging with modern imaging, advances in radiotherapy delivery, improved surgical techniques and introduction of novel targeted therapy, the cure rate for most HPV-negative HNSCC remains low with high treatment morbidity.

Hypoxia, the condition of low oxygen level, occurs frequently in HNSCC. It is a result of the imbalance between oxygen delivery and oxygen consumption within the tumor. There is abundant evidence that hypoxia induces resistance to radiation, which is a well-accepted treatment modality in HNSCC [6], [7]. Hypoxia has also been implicated in increased resistance to systemic therapy and enhanced nodal and distant tumor spread [8], [9]. Therefore, significant effort has been devoted to targeting hypoxia in combination with radiotherapy to overcome these adverse effects of low tumor oxygenation. Clinical trials have evaluated oxygen modifiers, including hyperbaric oxygen breathing, carbogen and nicotinamide, and hypoxic cell radiosensitizers, such as the nitroimidazole derivatives and hypoxic cell cytotoxins [7]. Unfortunately, many randomized trials have shown limited improvement on disease control and patient survival.

Interestingly, further analysis of these trials suggested that some tumors are in fact vulnerable to hypoxia modification and these hypoxia-targeting agents can be exploited to enhance radiation-induced cell killing. The future of hypoxia-targeted therapy in HNSCC lies in determining who would benefit most from such treatment and to identify more active novel agents with less toxicity. More specific hypoxia-related biomarkers in serum and tumor tissue and better hypoxia-imaging modalities are necessary to further stratify HPV-negative HNSCC based on tumor oxygenation for individualized care.

14.2 Hypoxia in Solid Tumors

Hypoxia in solid neoplasms is defined as tissue oxygen tension below normoxic levels of 40–60 mm Hg [10]. Covering a wide and dynamic range, tumor oxygen partial pressure (pO_2) as low as 2.5 mm Hg has been frequently reported. This state

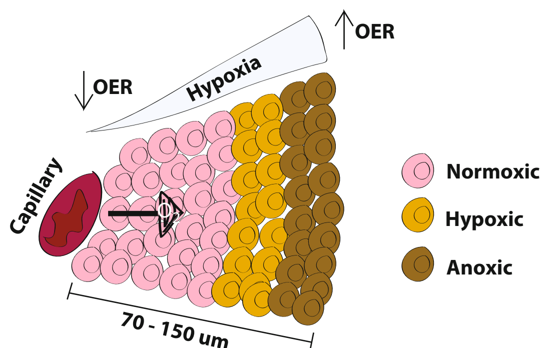

Fig. 14.1 Limitations in oxygen diffusion create regions of tumor hypoxia. The rapid metabolism rate of tumor cells impedes the distance at which oxygen can travel through the tissue. Regions within 70 μm of a capillary are well oxygenated (*pink*), whereas varying degrees of hypoxia exist beyond this distance [12], [13]. When oxygen is depleted (anoxic) tumors become necrotic (*brown*). Hypoxic cells fall between the two extremes in tumor oxygenation (*yellow*)

of reduced tumor oxygenation is a common occurrence in most solid tumors due to rapid cancer-cell proliferation with increased oxygen demand that cannot be met by the surrounding leaky vasculature. As a result, perfusion pressure, erratic blood flow, interstitial fluid leakage, and mechanical pressure from within the tumor and surrounding tissues, all serve as factors that interfere with tissue oxygenation. HNSCC arises from nonvascularized epithelium, which is relatively hypoxic under normal conditions. HNSCC may therefore possess enhanced survival potential under prolonged hypoxia in the tumor microenvironment.

Hypoxia does not typically occur in a homogeneous fashion, rather the level of oxygenation within a tumor is spatially and temporally heterogeneous. This phenomenon occurs, in part, due to changes in cellular oxygen consumption as the tumor expands along with the complex supporting vasculature. Temporal heterogeneity arises in instances of transient changes in cellular oxygen consumption, which can also be described as acute or perfusion-limited hypoxia. Under this circumstance, aberrant vasculature can cause fluctuations in blood flow including temporary shut off or even backflow from nonfunctional shunts. This phenomenon was first demonstrated using sequential injection of different fluorescent dyes 20 minutes apart into subcutaneous and intramuscular-implanted mouse tumors, revealing regions of unmatched staining, suggesting intermittent blood flow [11]. On the other hand, spatially heterogeneous, or chronic hypoxia, occurs due to limitations in oxygen diffusion from and paucity of blood vessels (Fig. 16.1) [12]. This was evident in histological samples, which showed that tumor necrosis (hypothesized to be a consequence of chronic hypoxia) consistently occurred around 100–150 μm from blood vessels [13].

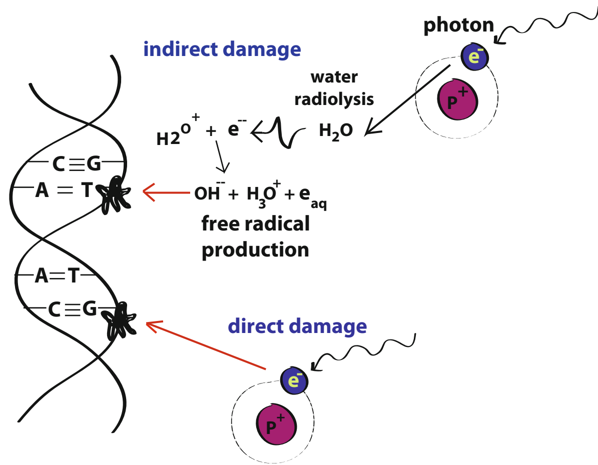

Fig. 14.2 Ionizing radiation causes direct and indirect DNA damage. The former arises from direct ionization of and electron transfer to the DNA. The latter occurs from ionization of water (radiolysis) to produce the hydroxyl radical, which can interact with the 2′-deoxyribose or nucleobases [14]

14.3 Hypoxia-induced Radiation Resistance

The necessity of oxygen for radiation cytotoxicity is the ultimate cause of hypoxia-induced radiation resistance. This relationship between oxygen and radiation sensitivity is among the most extensively studied effects in radiobiology. Cell kill by ionizing radiation occurs through direct and indirect DNA damage. For instance, DNA can be ionized by direct photo interaction. Alternatively, indirect DNA damage can be caused by secondary radicals. Ionizing radiation-induced water lysis leads to the production of H_2O_2 and hydroxyl radicals, which cause double stranded, single stranded, and base breaks, as well as DNA-protein cross-linking. In addition, sugar-radical production and chemical modification of purine and pyrimidine bases can also occur. Oxygen serves to stabilize these radiation-induced free radical species; therefore, DNA in close proximity to these free radicals undergoes increased damage events (Fig. 14.2) [14].

Over 50 years ago, Gray observed that a radiation dose of approximately three times greater was required to kill hypoxic cells compared to their well-oxygenated counterpart. This phenomenon is quantified as an oxygen enhancement ratio (OER) and reflects the effect of oxygen on cell survival after exposure to radiation. The OER is a ratio of the doses given under hypoxic and normoxic conditions that would yield the same biological effect; it typically falls between 2.7 and 3.0 and can transition from 1 to 3 when tumor oxygen tension is below 5 mm Hg. OER also depends on various factors such as the pO_2 of the hypoxic and normoxic conditions, cell/tissue type, radiation dose, and linear energy transfer (LET) of the radiation applied [15].

14.4 Tumor Hypoxia and Poor Prognosis in HNSCC

Most patients with locally advanced HNSCC require radiation therapy with or without chemotherapy as part of their treatment regimen either definitively or after surgery. Studies that related treatment outcomes to direct tumor pO_2 measurements

by needle electrodes have shown that tumor hypoxia is a major contributor to poor prognosis after radiotherapy [16]–[18]. Nordsmark and colleagues reported that tumor pO_2 below 2.5 mm Hg was an independent predictor for radiation response and locoregional tumor control in a large cohort of HNSCC on multivariate analysis [19]. Brizel and colleagues reported a significantly lower 12-month disease-free survival in patients with median tumor pO_2 below 10 mm Hg versus those with higher tumor pO_2. The relationship between tumor oxygenation and prognosis has also been demonstrated with endogenous hypoxia markers, such as hypoxia-inducible factors (HIFs) and carbonic anhydrase-IX (CAIX), and positron emission tomography (PET)-based hypoxia tracers [20]–[23].

In addition to modifying radiation response, hypoxia also encourages a more malignant phenotype by creating selective pressure biased for cells that can survive and proliferate in otherwise unviable conditions, low in oxygen and nutrients [24]. Under this environment, changes in gene expression occur, including upregulation of genes that control angiogenesis, cell proliferation, survival, apoptosis, glucose metabolism, invasion and metastasis [25]. One of the most extensively studied regulators of oxygen-responsive pathways is HIF-1, a transcription factor that binds hypoxia response elements (HREs) on at least 200 target genes [26]–[28]. The hypoxic environment also promotes genomic instability, facilitating oncogenic potential [29]. Furthermore, tumor cells that are inherently more radioresistant and cancer stem cells that possess greater clonogenic potential can drive repopulation and relapse after therapy (Fig. 14.3) [30].

14.5 Tumor Hypoxia Detection

The ability to detect and measure hypoxia is critical in improving HNSCC radiotherapy. The well-established and extensively studied relationship between low tumor oxygenation and radioresistance relied on tumor hypoxia detection with more invasive approaches, including pimonidazole staining of biopsied samples and direct measurement with oxygen sensing electrodes, in addition to noninvasive methods based on PET imaging of radiolabeled hypoxia tracers. Tumor hypoxia detection is also critical in clinical decision making. The merging of PET hypoxia marker imaging with CT anatomical information provides greater tumor volume resolution, which is essential for staging, radiotherapy planning, and evaluation of tumor response to treatment. Noninvasive hypoxia tracer imaging and standardized hypoxia biomarkers, circulating in the blood and/or expressed in the tumor, will be critical for patient selection.

14.5.1 Direct Measurement of Tumor Hypoxia

Previously considered the "gold standard" of tumor hypoxia measurement, direct measurement of tissue oxygenation was first conducted using polarographic needle electrodes (pO_2 histograph, Eppendorf, Hamburg, Germany) in cancers of the head

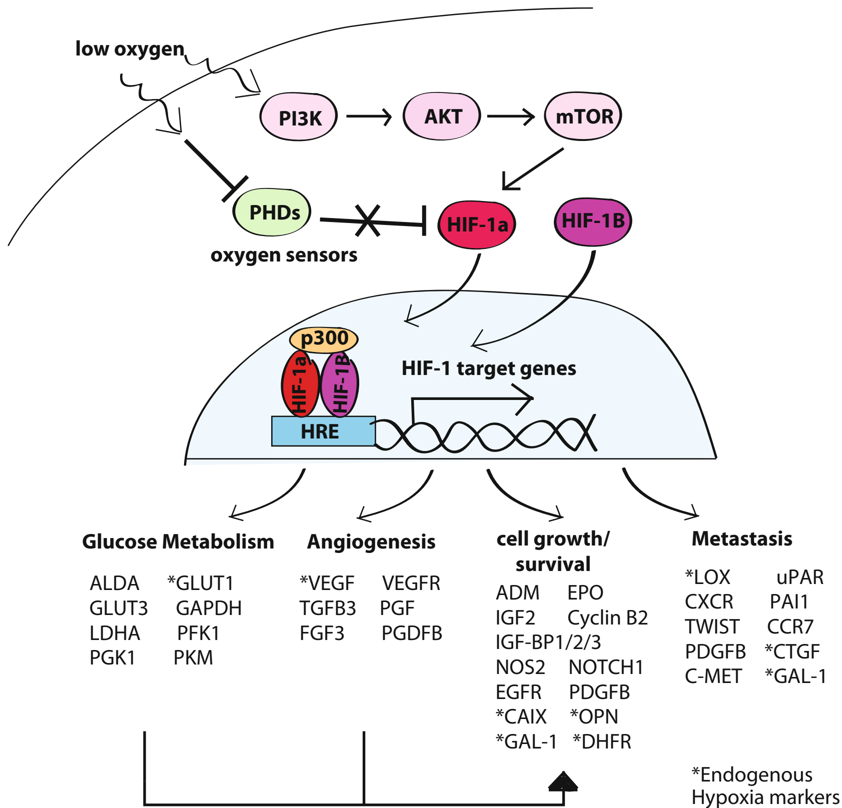

Fig. 14.3 Hypoxia-induced *HIF-1* signaling pathways. The HIF-1 transcription factor acts as a global regulator of hypoxia-responsive gene expression. Tumor hypoxia activates HIF-1 through *mTOR* signaling and inhibition of 4-prolyl hydoxylases (*PHDs*)-mediated degradiation. Once stabilized, HIF-1, a heterodimer consisting of alpha and beta subunits, and the *p300* transcriptional coactivator bind to and induce the expression of genes harboring a hypoxia response element (*HRE*) [26]–[28]. HIF-1 activation enables tumor cells to adapt and survive in otherwise growth-limiting environments through the modulation of a multitude of cellular processes including glucose metabolism, angiogenesis, growth, survival, and metastasis [25]

and neck, cervix, and breast. Several independent investigators subsequently showed that tumor oxygenation, measured by these probes, was associated with treatment outcome in HNSCC [17], [31]. This method involves an oxygen sensor placed at the tip of a needle that is positioned and inserted by ultrasound or computed tomography (CT) guidance and advanced through the tissue by a step motor. Rapid measurements can be made every 1.4 s to collect 50–80 measurements along 5–6 tracks to generate a histogram of pO_2 in tumor or tissue of interest. Notably, this approach has been validated by comparison of electrode measurements against exogenous and endogenous hypoxia marker staining of corresponding histological samples [32], [33].

Unfortunately, electrode techniques are not without limitations. Invasive by nature, probe insertion and travel may cause tissue damage. Measurements can only be made in superficial regions, rendering many deeply seated tumors such as those

in the brain, lung, and gastrointestinal (GI) tract inaccessible for direct evaluation. Variation in readings collected by different observers (interobserver variability) is unfortunately common and can affect final results. Finally, dynamic changes in oxygen tension throughout the tumor cannot be adequately resolved due to the inability to acquire spatially comprehensive readings over time. A variety of indirect methods for imaging exogenous and endogenous hypoxia markers have since been developed to address the limitations of probe measurement.

14.5.2 Indirect Measurement of Tumor Hypoxia

Numerous injectable metabolic and bioreductive-based hypoxia markers have been created to measure tumor hypoxia in a noninvasive manner. When radiolabeled with PET-compatible isotopes, these markers provide a means to image tumors in situ while capturing the temporal and spatial heterogeneity of hypoxia that direct probe measurement cannot perform. The merging of PET hypoxia marker imaging with CT anatomical information provides greater tumor volume resolution.

14.5.2.1 Bioreductive Probes

Hypoxia imaging with bioreductive 2-nitroimidazoles was initially proposed three decades ago and was developed as a more efficient compound with increased electron affinity [34]. Nitroimidazoles, which were originally created using tritiated misonidazole, undergo an enzymatic reduction to a radical anion that is back-oxidized to its starting compound in well-oxygenated conditions. However, in a hypoxic environment, the radical anion is further reduced and remains bound to macromolecules where it is irreversibly retained in hypoxic cells. Trapped nitroimidazole compounds are detected by specific antibodies for immunohistological analysis of biopsied tumor samples or radiolabeled for PET imaging in vivo.

Pimonidazole ([1-(alpha-methoxymethylethanol)-2-nitroimidazole]), which is frequently used in small animal tumor models, is administered at a nontherapeutic dose followed by specific antibody detection of pimonidazole adducts in hypoxic cells within frozen and fixed tumor samples [35], [36]. Kaanders and colleagues demonstrated that in patients receiving pimonidazole injection prior to HNSCC biopsy, the degree of exogenous hypoxia marker binding was significantly associated with locoregional control and disease-free survival after radiation treatment [29].

Fluorine 18-fluoromisonidazole (FMISO, [1-(2-nitroimidazolyl)-2-hydroxy-3-fluoropropane]) is one of the most widely used PET imaging tracer in head and neck cancer. Regions identified as hypoxic by FMISO are clinically significant, correlating with staining for CAIX and pimonidazole hypoxia markers [37]–[39]. Notably, FMISO accumulation corresponded with poor response to radiotherapy [23], [40]–[42]. Strong FMISO uptake has also been shown to predict worse outcome in HNSCC treated with tirapazimine (TPZ), a hypoxic cell cytotoxin, and chemoradiotherapy [43]. Due to slow specific accumulation in target tumor tissues and clearance kinetics from normoxic tissues, clinical application of FMISO

is limited and agents with higher signal to noise ratio are still in demand [44]. Exhibiting faster diffusion through cell membranes, accumulation in target hypoxic tumor cells and clearance from normoxic tissues, Fluoroazomycinarabinofuranoside (FAZA) is another nitroimidazole with sugar addition that generates better signal to noise ratio than FMISO [45], [46]. Furthermore, FAZA uptake also correlated with benefit from TPZ treatment [47]. EF5 ([2-(2-nitro-1H-imidazol-yl)-N-(2,2,3,3,3-pentaflouropropyl) acetamine]), a fluorinated derivative of the 2-nitroimidazole, Etanidazole, can be 18-F labeled for PET imaging and has been shown to predict radioresistance in individual tumors in murine and rat models [48]. Recently, Chitneni and colleagues described a novel simplified method of EF5 synthesis that meets the standards of purity and activity for clinical use, but is also amenable to automated synthesis, giving [(18)F] EF5 PET promise for clinic use [49].

Although a variety of bioreductive complexes are available to image tumor hypoxia, a requirement for novel hypoxia imaging agents exists in order to address the limitations of current bioreductive tracers. There is a great need for tracers that exhibit faster and more specific localization as well as rapid clearance from well-oxygenated tissues, boosting signal to noise ratio. Efforts towards improving hypoxia tracers has yielded compounds varying in their lipophilicities and biodistributions, several of which are in various stages of animal and clinical testing, including^{62}Cu-diacetyl-bis(N4-methylthiosemicarbazone (^{62}CU-ATSM) and 3-[F]fluoro-2-(4-((2-nitro-^{1}H-imidazol-1-yl)methyl)-1H-1,2,3,-triazol-1-yl)-propan-1-ol (F-HX4) [50], [44], [51].

14.5.2.2 Endogenous Hypoxia Markers

Endogenous hypoxia-related markers are defined as proteins whose level is increased by either upregulation or less degradation in the presence of hypoxia. Examination of hypoxia-responsive molecular markers is an alternative and cost-efficient method for assessing tumor oxygenation. Virtually all archived tumor samples are amenable to endogenous marker analysis, unlike exogenous markers that must be injected prior to biopsy. Notably, rapid comparison of hypoxia-related markers to treatment outcome can facilitate identification of markers with prognostic and/or predictive value. Since the HIF (HIF-1 and HIF-2) pathways for oxygen response are of the most extensively studied, downstream targets including CAIX, vascular endothelial growth factor (VEGF), and glucose transporter-1 (Glut1) have been examined for their prognostic capabilities in HNSCC, but with mixed results [52]–[54]. Gene expression and proteomic analysis are well-accepted approaches to identify additional endogenous hypoxia-responsive gene signatures, markers, and hypoxia metagenes. Through these approaches, HIF-regulated and non-HIF-regulated proteins such as connective tissue growth factor (CTGF), osteopontin (OPN), ephrin A1, hypoxia-inducible gene-2 (HIG2), dihydrofolate reductase (DHFR), galectin-1, IkappaB kinase beta (IKK-b), and lysyl oxidase (LOX), have been examined histologically in HNSCC to find markers that can predict cancer-specific survival, overall survival, and identify HNSCC patients best suited for hypoxia-targeted therapies [55]. However, the clinical relevance of these markers and others require further investigation and validation.

Several limitations exist for this method of tumor hypoxia assessment. For instance, several of these hypoxia-responsive genes may not be hypoxia specific as they can be induced or modulated by hypoxia-independent processes such as stimulation by nitric oxide, cytokines, and oncogenes [56], [57]. Staining and measurement of any endogenous proteins are assay and antibody dependent, and technical variability from different studies needs to be reconciled. Furthermore, staining patterns of certain endogenous markers can differ significantly from exogenous tracers, making it hard to determine the significance of either marker type. One thing for certain is that it is unlikely that a single endogenous biomarker can be used to reflect the temporal and spatial complexity of hypoxia and that a panel of hypoxia-responsive markers will be necessary to address the aforementioned limitations by increasing specificity.

14.6 Targeting Hypoxia to Improve Radiation Response

Great effort has been devoted to targeting hypoxia clinically in order to improve radiation response in solid tumors. Means to modify tumor oxygenation such as hyperbaric oxygen breathing (HBO) and the combination of carbogen and nicotinamide (ARCON) have been tried to increase oxygen delivery to tumors, thereby rendering them more radiosensitive. The use of hypoxic cell radiosensitizers (to sensitize hypoxic cell to radiation) and hypoxic cell cytotoxins (to directly kill hypoxic tumor cells) have similarly been tested in combination with either radiation or chemoradiation in multiple clinical trials in HNSCC with little success. Current efforts are devoted to identifying and targeting hypoxia-driven genes or pathways, specifically to impair the HIF pathway and tumor metabolism.

14.6.1 Tumor Oxygen Modifiers

The purpose of HBO is to boost oxygen delivery to tumors, thereby increasing their sensitivity to radiation. Patients breath 100 % oxygen under increased atmospheric pressure in order to facilitate oxygen diffusion into the tumor and circumvent diffusion-limited hypoxia. Several clinical trials were conducted to test the benefit of combined HBO and radiotherapy (RT) in HNSCC. A comparison of two separate studies with the same fractionation schedule and endpoints showed an improvement in the local control rates: 43 % for HBO/RT versus 16 % for RT in one study and 53 % for HBO/RT versus 30 % for RT in the other [58], [59]. The improvement in local control was primarily noted for smaller tumors but not for larger ones [58], [60]. Several groups also noted a significant increase in survival at 1 year in HNSCC patients receiving combined HBO and radiation, but not at 2 years [59], [61], [62]. Comparisons in benefit of HBO and RT were difficult to make since the various studies lacked consistency in hyperbaric pressure, radiation fractionation, and total dose given. A meta-analysis of combined HBO and RT revealed that significant reductions in deaths at 5 years were primarily found in patients who received 12 or

fewer fractions with HBO, making benefit from hypofractionation and HBO difficult to distinguish [63]. Furthermore, the use of HBO was associated with a higher rate of bone, soft tissue, and cartilage necrosis, especially when it is delivered with larger radiation fraction size [7]. Additional limitations for HBO include the patient's poor ability to tolerate a pressurized environment due to claustrophobia and occasional seizure, and the cumbersome process of HBO and RT set up [58], [64]. Because of these issues, combined HBO and RT has not been adopted in the clinic [65]. Ultimately, HBO studies have contributed more towards highlighting the significance of tumor hypoxia in radioresistance than improving tumor response to radiation.

Clinical trials for accelerated radiotherapy with carbogen and nicotinamide (ARCON) sought out to target tumor hypoxia by addressing both diffusion-limited hypoxia through carbogen hyperoxic gas breathing (inhalation of 98 % oxygen and 2 % carbon dioxide) and perfusion-limited hypoxia with a vasoactive agent, nicotinamide. A Phase III randomized trial was launched to determine improvement of tumor control with ARCON (64 Gy within 36–38 days with carbogen breathing 4 min before and during daily radiations and oral nicotinamide) compared to radiation alone (68 Gy within 36–38 days). Disappointingly, no significant difference was reported between the two treatment groups for the primary endpoint, which was local control (AR 80 % versus ARCON 83 % at 2 years, and AR 78 % versus ARCON 79 % at five years). Disease-free survival and overall survival also did not improve with ARCON treatment. Interestingly, a significant improvement was observed for regional control rate (AR 88 % versus ARCON 95 % at 2 years and AR 86 % versus ARCON 93 % at 5 years) [66]. It is unclear why ARCON treatment should improve nodal control. However, since nodal control was not the primary endpoint of the study, the patients were not stratified by nodal status and a multivariate analysis was not performed, this observation should be considered hypothesis generating and needs to be confirmed in another randomized trial.

14.6.2 Hypoxic Cell Radiosensitizers and Cytotoxins

Since the mid-1970s, approaches to circumvent hypoxia-induced treatment resistance in HNSCC have focused on utilizing hypoxic cell radiosensitizers in combination with radiation. These sensitizers were developed as electron-affinic compounds that selectively increase radiation-induced cell kill of hypoxic cells by mimicking the effect of oxygen. Research conducted with these hypoxia-specific radiosensitizers soon lead to the development of hypoxic cell cytoxins, which rather than sensitize, kill tumor cells with low oxygen tension. In particular, the 2-nitroimidazoles, including misonidazole, ethanidazole, and nimorazole, garnered great attention as dual-functionality hypoxic cell radiosensitizers and cytotoxins [67].Under hypoxia, these bioreductive agents are metabolized by intracellular reductases to form cytotoxic reactive radical species that induce cell death by single and double-stranded DNA breaks and chromosomal aberrations [68].

14.6.2.1 Nitroimidazole Studies

Since the mid-1970s, nitroimidazoles were the main agent used in the clinic to target tumor hypoxia. Multiple clinical trials conducted by the Radiation Therapy Oncology Group (RTOG), the European Organization for Research and Teaching of Cancer (EORTC) and the Danish Head and Neck Cancer Group (DAHANCA), investigated the benefit of combining a variety of 2-nitroimidazole derivatives with radiation, only to report disappointing findings. Between 1979 and 1985, the EORTC investigated a split-course accelerated fractionation regimen with or without misonidazole compared to standard fractionation in patients with locally advanced HNSCC. Unfortunately, shortening the radiation course with an interruption mid course and misonidazole did not improve locoregional control or survival after 5 years [69]. RTOG conducted a randomized Phase III trial to compare the combination of etanidazole (2.0 g/m^2 three times a week for 17 weeks) and conventional RT (range: 66 Gy in 33 fractions to 74 Gy in 37 fractions) to RT alone. The addition of etanidazole to radiation therapy failed to provide benefit [70]. Similarly negative results were reported by a European Etanidazole trial [71]. Finally, although less potent in inducing radiosensitization than etanidazole, a less toxic compound, Nimorazole (1-(N-B-theylmorpholine)-5-nitro-imidazole), was tested in a randomized double-blind Phase III study by the DAHANCA group. Patients received conventional RT to a total dose of 62–68 Gy (2 Gy/fraction, five fractions per week) with or without nimorazole. Although the addition of nimorazole did not significantly affect overall survival, improvements were observed for both 5-year loco-regional control (49 % nimorazole versus 33 % placebo) and cancer related deaths (52 % nimorazole versus 41 % placebo) [72]. To date, nimorazole is the only nitroimidazole hypoxic cell radiosensitizer that is being used in the clinic.

14.6.2.2 Tirapazamine Studies

Tirapazamine (TPZ), is an aromatic heterocycle di-N-oxide (3-amino-1,2,4-benzotriazine-1,4 dioxide) that was developed as a hypoxic cell cytotoxin. It has also been evaluated in clinical trials in combination with chemotherapy and radiation in HNSCC. The preclinical observation that TPZ was highly active in mammalian cells in vitro when combined with fractionated radiation at doses comparable to those used in the clinic sparked interest in investigating its therapeutic potential in various cancers including HNSCC [73]. Ample preclinical evidence supported TPZ antitumor activity. Dorie and Brown reported that the combination of TPZ and various chemotherapy agents had an additive antitumor effect in an implanted fibrosarcoma mouse model [74]. Interestingly, mounting evidence suggested that the additive effect of TPZ and platinum-based chemotherapy is hypoxia-dependent [75]. Furthermore, in an uncontrolled Phase II trial, TPZ combined with radiation resulted in a promising local control rate at 2 years when compared to historical results [76]. In a randomized Phase II trial (TROG 98.02), the addition of TPZ improved the 3-year failure-free survival and locoregional failure-free rates (55 and 84 %) compared to radiation and chemotherapy only (44 and 66 %) [77].

Based on the promising Phase II trial results, TROG 02.02 (headSTART) randomized Phase III clinical trial was launched to investigate the benefit of adding TPZ to a concurrent radiation and cisplatin regimen in locally advanced HNSCC. This study involved 88 centers from 13 countries and 861 patients with stage III and IV tumors, affecting the oral cavity, oropharynx, hypopharynx, and larynx. Patients received radiation (70 Gy over 7 weeks) and either Cisplatin (100 mg/m^2/d on day 1 of weeks 1, 4, and 7) or Cisplatin (75 mg/m^2/d on day 1 of weeks 1, 4, and 7), and TPZ (290 mg/m^2/d on day 1 of weeks 1, 4, and 7, and 160 mg/m^2 on day 1, 3, and 5 of weeks 2 and 3). Disappointingly, TPZ addition did not improve 2-year overall survival [78]. The failure to show benefit of TPZ in this trial may be due to the significant number (25 %) of patients deviating from the prescribed radiation treatment. When these patients were excluded from analysis, TPZ treatment was associated with higher locoregional control. Notably, analysis of tumor hypoxia in parallel to outcome suggested the importance of patient pretherapeutic evaluation, discussed below.

14.6.3 HIF-targeted Therapies

HIF-1 is a key transcriptional regulator of hypoxia-responsive genes and targeting HIF-1 has become a compelling method for circumventing hypoxia-mediated tumor aggressiveness and radioresistance. Although targeting HIF-1 directly is challenging, inhibition of upstream and downstream pathways are attractive approaches. Numerous methods have been developed to inhibit HIF-1 protein accumulation (at the transcriptional, translational, and degradation levels) and transcriptional activity (by blocking its ability to bind DNA). For example, HIF-1 translation can be inhibited by targeting the PI3K/AKT/mTOR pathway through mTOR (temsirolimus, rapamycin and rapamycin analogs (Rad001 and CCI-799)) and PI3K inhibition (LY294002). HIF-1 induction can also be attenuated through the perturbation of epidermal growth factor receptor (EGFR) signaling pathways with small molecule inhibitors (gefitnib, erlotinib) and blocking antibodies (cetuximab, panitumumab) [79], [80]. Furthermore, Adachi and colleagues demonstrated that inhibition of STAT3-mediated HIF-1 translation attenuated murine orthotopic HNSCC growth [81]. Chemical inhibitors have also been developed to block HIF-1a protein accumulation, including 2-methoxyestradiol (2ME2) and 2-phenethyl isothiocynate (PEITC), which block HIF-1a translation and nuclear translocation. HIF-1a protein degradation can be induced through inhibition of Hsp90, a stabilizing chaperone, with inhibitors including, 17-N-Allylamino-17-demethoxy geldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), and geldanamycin [79], [82]. HIF-1 degradation can also be induced by histone deacetylase (HDAC) inhibitors, Trichostatin A (TSA) and hydroxamic agent, LAQ824, through acetylation and polyubiquitination. S-2-amino-3-[40-N, N,-bis(2-chloroethyl)amino] (PX-478), derived from mephalan, inhibits HIF-1α on multiple fronts, blocking its transcription, translation and promotes its degradation [83]. A multitude of agents also block HIF-1 function by preventing its DNA binding and

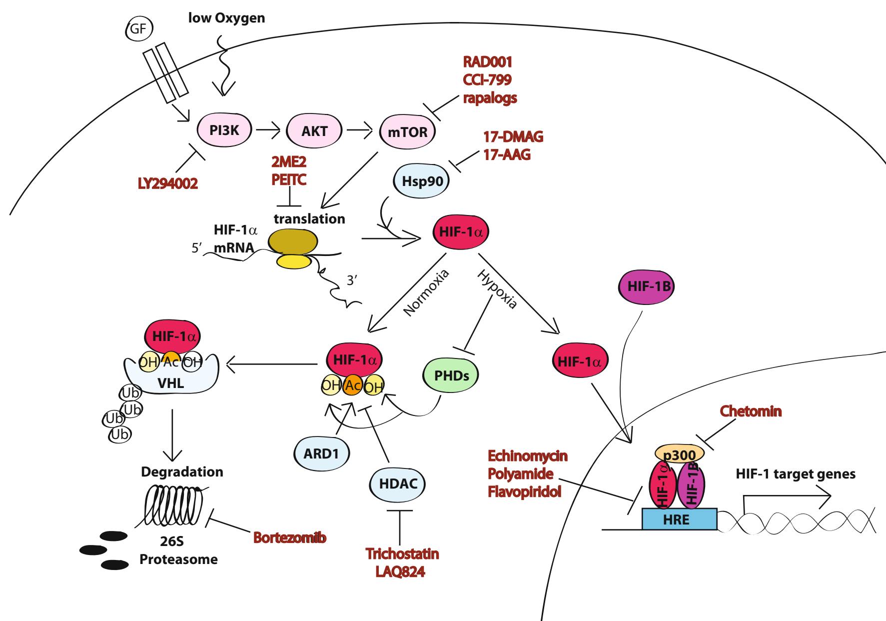

Fig. 14.4 Inhibitors of *HIF-1*. As a mediator of tumor aggressiveness and radiation resistance, the inhibition of HIF-1 has become a desirable strategy to target hypoxic tumors. Numerous inhibitors have been developed to block HIF-1, including the attenuation of protein stability and accumulation (17-AAG, 17-DMAG, Trichostatin, LAQ824, RAD001, CCI-799, rapalogs, LY294002, 2ME2, PEITC) as well as *HRE* binding and transcriptional activity (Echinomycin, Polyamide, Flavorpiridol, Chetomin) [79], [80], [82], [83]

transcriptional activation of target genes. These compounds include echinomycin, polyamides and flavopiridol, which prevent HIF-1α interaction with hypoxia response elements (HREs). HIF-1α transcriptional activation can be abrogated by targeting its coactivator p300 directly with chetomin or disrupting their interaction with the proteasome inhibitor, bortezomib (Fig. 14.4) [80].

Inhibition of HIF-1 activity downstream by targeting genes that regulate glycolysis may significantly attenuate tumor aggressiveness since this unique metabolic profile promotes radioresistance, tumor-cell migration, angiogenesis, and evasion from immune surveillance [84], [85]. Cairns and colleagues have shown that inhibition of pyruvate dehydrogenase kinase 1 (PDK1), which limits pyruvate entry into the citric acid cycle and reduces mitochondrial oxygen consumption, causes a transient increase in tumor hypoxia. The temporary reduction in tumor oxygenation can be exploited with hypoxic cell cytotoxins including TPZ, which are more effective under this condition [86].

14.7 The Future of Hypoxia Targeting in HNSCC: The Importance of Patient Selection

14.7.1 Tumor Hypoxia Pre-evaluation

Clinical trials that evaluated hypoxic cell cytotoxins or radiosensitizers in HNSCC have indicated that some tumors have modifiable hypoxia while others do not. Therefore, the future of hypoxia targeting in HNSCC will need to incorporate pretreatment evaluation of tumor oxygenation. The ideal approaches for patient selection would be noninvasive, reproducible at different institutions and validated prospectively. Some of these approaches may include hypoxia imaging or detection of hypoxia-related markers or both.

Rischin and colleagues conducted a substudy within the previously mentioned TROG Phase II study (TROG 98.02), in which a subset of patients received FMISO hypoxia imaging before and at mid-treatment. Patients with high FMISO uptake within the primary tumor (hypoxic tumors) had a greater locoregional failure rate when treated with chemoradiation alone compared to those treated with chemoradiation and TPZ [43]. These results suggested a role for FMISO imaging in identifying patients who would benefit from TPZ. However, the number of patients involved in this substudy was quite small (45 patients in total) and these findings need to be validated in a larger independent trial.

Another translational side study used pimonidazole staining from tumor biopsies to assess tumor hypoxia in larynx cancers and correlated tumor response to ARCON treatment based on this marker staining [66]. They found that hypoxic tumors by pimoninidazole staining ($> 2.6\,\%$ positive staining in the tumor) responded significantly better to ARCON compared to accelerated radiotherapy (AR) alone. In addition, significant improvement in regional control was achieved with ARCON in these patients (AR 55 % versus ARCON 100 %, $p = 0.01$) whereas patients with less hypoxic tumors did not experience any improvement (AR 96 % versus ARCON 92 %). Similarly, there was a trend for improvement in 5-year disease-free survival with ARCON therapy in patients with hypoxic tumors (AR 40 % versus ARCON 86 %, $p = 0.08$), a benefit that was not seen in patients with better oxygenated tumors [22], [66]. Although these data are intriguing, the number of patients with pimonidazole assessment was small (79 patients in total, 26 with hypoxic tumor) and will need validation from a larger independent study. Nevertheless, the results of these two cited studies suggested that noninvasive tumor hypoxia assessment with 2-nitroimidazole tracers may assist in selecting patients who will respond to hypoxia-modifying therapies.

Zips and colleagues conducted a FMISO imaging study that highlighted the significance of tumor oxygenation dynamics during RT when defining a time point for hypoxia imaging with greatest predictive value. Imaging with FMISO-PET/CT at earlier time points during radiation (weeks 1 and 2 or 10 to 20 Gy) served as stronger indicators of local progression-free survival. In an exploratory cohort of patients, tumors with greater tracer uptake that did not resolve to or below baseline values by the

20 Gy time point were at a greater risk of local recurrence, albeit with small patient numbers and limited statistical power [22].These findings also support the value of noninvasive tumor hypoxia in patient selection for more aggressive treatments involving dose intensification or hypoxia-modifying therapies.

14.7.2 Tumor HPV Status in Patient Selection

A subset of HNSCCs, specifically oropharyngeal carcinoma (OPC), are associated with high-risk human papilloma virus-16 and 18 (HPV-16 and 18) infection and are more responsive to radiation and chemotherapy. Patients with HPV(+) OPC are known to have superior prognosis than those with HPV(-) OPC as demonstrated in both retrospective and prospective studies [87]–[95], [4]. The incidence of HPV(+) OPC has been increasing rapidly in the western hemisphere in the last 30 years. Epidemiologically, these patients are typically younger, have minimal cigarette or alcohol consumption, and higher performance status when compared to HPV(−) tumors. The mechanism by which HPV(+) tumors are more responsive to radiation is not well understood but has been hypothesized to be related to better immune surveillance to viral-specific antigens, intact p53 function, and less overall genomic stability compared to HPV(−) tumors [96], [97].

An important question that needs to be addressed is whether HPV(+) and (−) OPC harbor the same level of hypoxia and whether hypoxia modification has a similar impact for both tumor types. The first question is being addressed through hypoxia imaging in a clinical study at Memorial Sloan–Kettering Cancer Center (personal communication, Nancy Lee, MD). The second question was addressed by reanalyses of prior hypoxia-targeted clinical trials. Rischin and colleagues showed a trend for improved locoregional control in HPV(−) OPC, but not in the HPV(+) group in the above mentioned TROG 02.02 Tirapazamine study [95]. Similarly, a secondary analysis of the DAHANCA nimorazole Phase III trial revealed that the addition of nimorazole only benefited patients with HPV(−) tumors but not those with HPV(+) tumors [88]. These data suggested that hypoxia modification is unlikely to benefit HPV(+) patients, who enjoy extremely good prognosis with conventional therapy. Therefore, future trials will be taking tumor HPV status into account and hypoxia targeting should primarily focus in patients with HPV(−) tumors where hypoxia modification may matter.

14.8 Conclusions

Acute and chronic hypoxia occur frequently in solid neoplasms of the head and neck and are major contributors to RT failure and poor prognosis in HNSCC. Hypoxia-induced radiation resistance is a well-established radiobiological phenomenon, which occurs as a result of fewer radiation-induced double-stranded DNA

breaks in a low oxygen environment. In turn, targeting hypoxia has been a compelling approach to circumventing radioresistance in head and neck tumors. Despite promising preclinical data, evidence of clinical benefit from hypoxia targeting in patients was often obscured in the absence of appropriate patient selection. Secondary studies revealed that some tumors were in fact amenable to hypoxia modification therapies; however, there is no present uniformed agreement regarding the best approach to identify these tumors. In order for hypoxia targeting to be successful in the clinics, we will need to develop a reliable sensitive and noninvasive method to assess tumor hypoxia in situ in human tumor for patient selection into clinical trials. Future trials will also need to take into account important established prognostic factors such as tumor HPV status to avoid inclusion of patients who do not benefit from hypoxia-targeted therapy.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–108.
2. Brizel DM. Targeting the future in head and neck cancer. Lancet Oncol. 2009;10:204–5.
3. Li XM, Di B, Shang YD, Zhou YQ, Ma HM, et al. [Analysis of risk factors in the prediction of distant metastases of head and neck squamous cell carcinomas]. Zhonghua Er Bi Yan Hou Ke Za Zhi. 2004;39:171–5.
4. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363:24–35.
5. Bourhis J, Overgaard J, Audry H, Ang KK, Saunders M, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. Lancet. 2006;368:843–54.
6. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OC. Concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol. 1953;26:638–48.
7. Overgaard J. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck-a systematic review and meta-analysis. Radiother Oncol. 2011;100:22–32.
8. Shannon AM, Bouchier-Hayes DJ, Condron CM, Toomey D. Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treat Rev. 2003;29:297–307.
9. Becker A, Hansgen G, Bloching M, Weigel C, Lautenschlager C, et al. Oxygenation of squamous cell carcinoma of the head and neck: comparison of primary tumors, neck node metastases, and normal tissue. Int J Radiat Oncol Biol Phys. 1998;42:35–41.
10. Brown JM. Tumor microenvironment and the response to anticancer therapy. Cancer Biol Ther. 2002;1:453–8.
11. Trotter MJ, Chaplin DJ, Durand RE, Olive PL. The use of fluorescent probes to identify regions of transient perfusion in murine tumors. Int J Radiat Oncol Biol Phys. 1989;16:931–4.
12. Brown JM. Tumor hypoxia, drug resistance, and metastases. J Natl Cancer Inst. 1990;82:338–9.
13. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer. 1955;9:539–49.
14. Karam PA, Leslie SA, Anbar A. The effects of changing atmospheric oxygen concentrations and background radiation levels on radiogenic DNA damage rates. Health Phys. 2001;81:545–53.
15. Wenzl T, Wilkens JJ. Modelling of the oxygen enhancement ratio for ion beam radiation therapy. Phys Med Biol. 2011;56:3251–68.
16. Nordsmark M, Overgaard M, Overgaard J. Pretreatment of oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol. 1996;41:31–40.
17. Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys. 1997;38:285–189.

18. Rudat V, Stadler P, Becker A, Vanselow B, Dietz A, et al. Predictive value of the tumor oxygenation by means of pO2 histography in patients with advanced head and neck cancer. Strahlenther Onkol. 2001;177:462–8.
19. Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol. 1996;41:31–9.
20. Koukourakis MI, Bentzen SM, Giatromanolaki A, Wilson GD, Daley FM, et al. Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. J Clin Oncol. 2006;24:727–35.
21. Ferreira MB, De Souza JA, Cohen EE. Role of molecular markers in the management of head and neck cancers. Curr Opin Oncol. 2011;23:259–64.
22. Zips D, Zophel K, Abolmaali N, Perrin R, Abramyuk A, et al. Exploratory prospective trial of hypoxia-specific PET imaging during radiochemotherapy in patients with locally advanced head-and-neck cancer. Radiother Oncol. 2012.
23. Kikuchi M, Yamane T, Shinohara S, Fujiwara K, Hori SY, et al. 18F-fluoromisonidazole positron emission tomography before treatment is a predictor of radiotherapy outcome and survival prognosis in patients with head and neck squamous cell carcinoma. Ann Nucl Med. 2011;25:625–33.
24. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 1996;379:88–91.
25. Le QT, Denko NC, Giaccia AJ. Hypoxic gene expression and metastasis. Cancer Metastasis Rev. 2004;23:293–310.
26. Semenza GL. (2007) Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE. 2007:cm8.
27. Takenaga K. Angiogenic signaling aberrantly induced by tumor hypoxia. Front Biosci. 2011;16:31–48.
28. Harris AL. Hypoxia-a key regulatory factor in tumour growth. Nat Rev Cancer. 2002;2:38–47.
29. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol. 2004;14:198–206.
30. Moncharmont C, Levy A, Gilormini M, Bertrand G, Chargari C, et al. Targeting a cornerstone of radiation resistance: cancer stem cell. Cancer Lett. 2012;322:139–47.
31. Nordsmark M, Overgaard J. A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. Radiother Oncol. 2000;57:39–43.
32. Nordsmark M, Eriksen JG, Gebski V, Alsner J, Horsman MR, et al. Differential risk assessments from five hypoxia specific assays: The basis for biologically adapted individualized radiotherapy in advanced head and neck cancer patients. Radiother Oncol. 2007.
33. Le QT, Sutphin PD, Raychaudhuri S, Yu SC, Terris DJ, et al. Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res. 2003;9:59–67.
34. Rasey JS, Grunbaum Z, Magee S, Nelson NJ, Olive PL, et al. Characterization of radiolabeled fluoromisonidazole as a probe for hypoxic cells. Radiat Res. 1987;111:292–304.
35. Graves EE, Vilalta M, Cecic IK, Erler JT, Tran PT, et al. Hypoxia in models of lung cancer: implications for targeted therapeutics. Clin Cancer Res. 2010;16:4843–52.
36. Young RJ, Moller A. Immunohistochemical detection of tumour hypoxia. Methods Mol Biol. 2010;611:151–9.
37. Troost EG, Laverman P, Philippens ME, Lok J, van der Kogel AJ, et al. Correlation of [18F]FMISO autoradiography and pimonidazole [corrected] immunohistochemistry in human head and neck carcinoma xenografts. Eur J Nucl Med Mol Imaging. 2008;35:1803–11.
38. Troost EG, Laverman P, Kaanders JH, Philippens M, Lok J, et al. Imaging hypoxia after oxygenation-modification: comparing [18F]FMISO autoradiography with pimonidazole immunohistochemistry in human xenograft tumors. Radiother Oncol. 2006;80:157–64.
39. Dubois L, Landuyt W, Haustermans K, Dupont P, Bormans G, et al. Evaluation of hypoxia in an experimental rat tumour model by [(18)F]fluoromisonidazole PET and immunohistochemistry. Br J Cancer. 2004;91:1947–54.

40. Rajendran JG, Schwartz DL, O'Sullivan J, Peterson LM, Ng P, et al. Tumor hypoxia imaging with [F-18] fluoromisonidazole positron emission tomography in head and neck cancer. Clin Cancer Res. 2006;12:5435–41.
41. Eschmann SM, Paulsen F, Reimold M, Dittmann H, Welz S, et al. Prognostic impact of hypoxia imaging with 18F-misonidazole PET in non-small cell lung cancer and head and neck cancer before radiotherapy. J Nucl Med. 2005;46:253–60.
42. Dirix P, Vandecaveye V, De Keyzer F, Stroobants S, Hermans R, et al. Dose painting in radiotherapy for head and neck squamous cell carcinoma: value of repeated functional imaging with (18)F-FDG PET, (18)F-fluoromisonidazole PET, diffusion-weighted MRI, and dynamic contrast-enhanced MRI. J Nucl Med. 2009;50:1020–7.
43. Rischin D, Hicks RJ, Fisher R, Binns D, Corry J, et al. Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: a substudy of Trans-Tasman Radiation Oncology Group Study 98.02. J Clin Oncol. 2006;24:2098–104.
44. Chen L, Zhang Z, Kolb HC, Walsh JC, Zhang J, et al. (1)(8)F-HX4 hypoxia imaging with PET/CT in head and neck cancer: a comparison with (1)(8)F-FMISO. Nucl Med Commun. 2012;33:1096–102.
45. Piert M, Machulla HJ, Picchio M, Reischl G, Ziegler S, et al. Hypoxia-Specific Tumor Imaging with 18F-Fluoroazomycin Arabinoside. J Nucl Med. 2005;46:106–13.
46. Postema EJ, McEwan AJ, Riauka TA, Kumar P, Richmond DA, et al. Initial results of hypoxia imaging using 1-alpha-D:-(5-deoxy-5-[18F]-fluoroarabinofuranosyl)-2-nitroimidazole (18F-FAZA). Eur J Nucl Med Mol Imaging. 2009;36:1565–73.
47. Rischin D, Fisher R, Peters L, Corry J, Hicks R. Hypoxia in head and neck cancer: studies with hypoxic positron emission tomography imaging and hypoxic cytotoxins. Int J Radiat Oncol Biol Phys. 2007;69:S61–S3.
48. Evans SM, Joiner B, Jenkins WT, Laughlin KM, Lord EM, et al. Identification of hypoxia in cells and tissues of epigastric 9 L rat glioma using EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide]. Br J Cancer. 1995;72:875–82.
49. Chitneni SK, Bida GT, Dewhirst MW, Zalutsky MR. A simplified synthesis of the hypoxia imaging agent 2-(2-Nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-[(18)F]pentafluoropropyl)-acetamide ([(18)F]EF5). Nucl Med Biol. 2012;39:1012–8.
50. Bourgeois M, Rajerison H, Guerard F, Mougin-Degraef M, Barbet J, et al. Contribution of [64Cu]-ATSM PET in molecular imaging of tumour hypoxia compared to classical [18F]-MISO-a selected review. Nucl Med Rev Cent East Eur. 2011;14:90–5.
51. Dubois LJ, Lieuwes NG, Janssen MH, Peeters WJ, Windhorst AD, et al. Preclinical evaluation and validation of [18F]HX4, a promising hypoxia marker for PET imaging. Proc Natl Acad Sci U S A. 2011;108:14620–5.
52. Vordermark D, Brown JM. Endogenous markers of tumor hypoxia predictors of clinical radiation resistance? Strahlenther Onkol. 2003;179:801–11.
53. Brockton N, Dort J, Lau H, Hao D, Brar S, et al. High stromal carbonic anhydrase IX expression is associated with decreased survival in P16-negative head-and-neck tumors. Int J Radiat Oncol Biol Phys. 2011;80:249–57.
54. Hong DY, Lee BJ, Lee JC, Choi JS, Wang SG, et al. Expression of VEGF, HGF, IL-6, IL-8, MMP-9, Telomerase in Peripheral Blood of Patients with Head and Neck Squamous Cell Carcinoma. Clin Exp Otorhinolaryngol. 2009;2:186–92.
55. Le QT, Kong C, Lavori PW, O'Byrne K, Erler JT, et al. Expression and Prognostic Significance of a Panel of Tissue Hypoxia Markers in Head-and-Neck Squamous Cell Carcinomas. Int J Radiat Oncol Biol Phys. 2007;69:167–75.
56. Brix B, Mesters JR, Pellerin L, Johren O. Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1alpha-mediated target gene activation. J Neurosci. 2012;32:9727–35.
57. Qian D, Lin HY, Wang HM, Zhang X, Liu DL, et al. Involvement of ERK1/2 pathway in TGF-beta1-induced VEGF secretion in normal human cytotrophoblast cells. Mol Reprod Dev. 2004;68:198–204.

58. Henk JM, Kunkler PB, Smith CW. Radiotherapy and hyperbaric oxygen in head and neck cancer. Final report of first controlled clinical trial. Lancet. 1977;2:101–3.
59. Haffty BG, Hurley R, Peters LJ. Radiation therapy with hyperbaric oxygen at 4 atmospheres pressure in the management of squamous cell carcinoma of the head and neck: results of a randomized clinical trial. Cancer J Sci Am. 1999;5:341–7.
60. Henk JM. Late results of a trial of hyperbaric oxygen and radiotherapy in head and neck cancer: a rationale for hypoxic cell sensitizers? Int J Radiat Oncol Biol Phys. 1986;12:1339–41.
61. Sealy R, Cridland S, Barry L, Norris R. Irradiation with misonidazole and hyperbaric oxygen: final report on a randomized trial in advanced head and neck cancer. Int J Radiat Oncol Biol Phys. 1986;12:1343–6.
62. Tobin DA, Vermund H. A randomized study of hyperbaric oxygen as an adjunct to regularly fractionated radiation therapy for clinical treatment of advanced neoplastic disease. Am J Roentgenol Radium Ther Nucl Med. 1971;111:613–21.
63. Bennett M, Feldmeier J, Smee R, Milross C. Hyperbaric oxygenation for tumour sensitisation to radiotherapy: a systematic review of randomised controlled trials. Cancer Treat Rev. 2008;34:577–91.
64. Giebfried JW, Lawson W, Biller HF. Complications of hyperbaric oxygen in the treatment of head and neck disease. Otolaryngol Head Neck Surg. 1986;94:508–12.
65. Bennett MH, Feldmeier J, Smee R, Milross C. Hyperbaric oxygenation for tumour sensitisation to radiotherapy. Cochrane Database Syst Rev. 2012;4:CD005007.
66. Janssens GO, Rademakers SE, Terhaard CH, Doornaert PA, Bijl HP, et al. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. J Clin Oncol. 2012;30:1777–83.
67. Cole S, Stratford IJ, Adams GE, Fielden EM, Jenkins TC. Dual-function 2-nitroimidazoles as hypoxic cell radiosensitizers and bioreductive cytotoxins: in vivo evaluation in KHT murine sarcomas. Radiat Res. 1990;124:S38–43.
68. Walton MI, Wolf CR, Workman P. Molecular enzymology of the reductive bioactivation of hypoxic cell cytotoxins. Int J Radiat Oncol Biol Phys. 1989;16:983–6.
69. Van den Bogaert WS, Schraub S, et al. The EORTC randomized trial on three fractions per day and misonidazole (trial no. 22811) in advanced head and neck cancer: long-term results and side effects. Radiother Oncol. 1995;35:91–9.
70. Lee DJ, Cosmatos D, Marcial VA, Fu KK, Rotman M, et al. Results of an RTOG phase III trial (RTOG 85-27) comparing radiotherapy plus etanidazole with radiotherapy alone for locally advanced head and neck carcinomas. Int J Radiat Oncol Biol Phys. 1995;32:567–76.
71. Eschwege F, Sancho-Garnier H, Chassagne D, Brisgand D, Guerra M, et al. Results of a European randomized trial of Etanidazole combined with radiotherapy in head and neck carcinomas [see comments]. Int J Radiat Oncol Biol Phys. 1997;39:275–81.
72. Overgaard J, Hansen HS, Overgaard M, Bastholt L, Berthelsen A, et al. A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. Radiother Oncol. 1998;46:135–46.
73. Zeman EM, Brown JM, Lemmon MJ, Hirst VK, Lee WW. SR 4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. Int J Radiat Oncol Biol Phys. 1986;12:1239–42.
74. Dorie MJ, Brown JM. Modification of the antitumor activity of chemotherapeutic drugs by the hypoxic cytotoxic agent tirapazamine. Cancer Chemother Pharmacol. 1997;39:361–6.
75. Beck R, Roper B, Carlsen JM, Huisman MC, Lebschi JA, et al. Pretreatment 18F-FAZA PET predicts success of hypoxia-directed radiochemotherapy using tirapazamine. J Nucl Med. 2007;48:973–80.
76. Lee D, Trotti A, Spencer S, Rostock R, Fisher C, et al. Concurrent tirapazamine and radiotherapy for advanced head and neck carcinomas: a phase II study. Int J Radiat Oncol Biol Phys. 1998; 42:811–15.
77. Rischin D, Peters L, Fisher R, Macann A, Denham J, et al. Tirapazamine, Cisplatin, and Radiation versus Fluorouracil, Cisplatin, and Radiation in patients with locally advanced head and neck cancer: a randomized phase II trial of the Trans-Tasman Radiation Oncology Group (TROG 98.02). J Clin Oncol. 2005;23:79–87.

78. Rischin D, Peters L, O'Sullivan B, Giralt J, Yuen K, et al. Phase III study of tirapazamine, cisplatin and radiation versus cisplatin and radiation for advanced squamous cell carcinoma of the head and neck. J Clin Oncol. 2008;26:abstr LBA6008.
79. Lee NY, Le QT. New developments in radiation therapy for head and neck cancer: intensity-modulated radiation therapy and hypoxia targeting. Semin Oncol. 2008;35:236–50.
80. Hu Y, Liu J, Huang H. Recent agents targeting HIF-1alpha for cancer therapy. J Cell Biochem. 2012.
81. Adachi M, Cui C, Dodge CT, Bhayani MK, Lai SY. Targeting STAT3 inhibits growth and enhances radiosensitivity in head and neck squamous cell carcinoma. Oral Oncol. 2012.
82. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. Nat Rev Drug Discov. 2003;2:803–11.
83. Welsh S, Williams R, Kirkpatrick L, Paine-Murrieta G, Powis G. Antitumor activity and pharmacodynamic properties of PX-478, an inhibitor of hypoxia-inducible factor-1alpha. Mol Cancer Ther. 2004;3:233–44.
84. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3:177–85.
85. Meijer TW, Kaanders JH, Span PN, Bussink J. Targeting Hypoxia, HIF-1, and Tumor Glucose Metabolism to Improve Radiotherapy Efficacy. Clin Cancer Res. 2012;18:5585–94.
86. Cairns RA, Papandreou I, Sutphin PD, Denko NC. Metabolic targeting of hypoxia and HIF1 in solid tumors can enhance cytotoxic chemotherapy. Proc Natl Acad Sci U S A. 2007;104: 9445–50.
87. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, et al. Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck. J Clin Oncol. 2009;27:1992–8.
88. Lassen P, Eriksen JG, Hamilton-Dutoit S, Tramm T, Alsner J, et al. HPV-associated p16-expression and response to hypoxic modification of radiotherapy in head and neck cancer. Radiother Oncol. 2010;94:30–5.
89. Lassen P, Eriksen JG, Krogdahl A, Therkildsen MH, Ulhoi BP, et al. The influence of HPV-associated p16-expression on accelerated fractionated radiotherapy in head and neck cancer: evaluation of the randomised DAHANCA 6 & 7 trial. Radiother Oncol. 2011;100:49–55.
90. Kong CS, Narasimhan B, Cao H, Kwok S, Erickson JP, et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys. 2009;74:553–61.
91. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008;100:261–9.
92. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, et al. Molecular classification identifies a subset of human papillomavirus-associated oropharyngeal cancers with favorable prognosis. J Clin Oncol. 2006;24:736–47.
93. Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst. 2008;100:407–20.
94. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92:709–20.
95. Rischin D, Young RJ, Fisher R, Fox SB, Le QT, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol. 2010;28:4142–8.
96. Snow AN, Laudadio J. Human papillomavirus detection in head and neck squamous cell carcinomas. Adv Anat Pathol. 2010;17:394–403.
97. Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. J Clin Oncol. 2006;24:2606–11.

Chapter 15
Sequencing HNC: Emergence of Notch Signaling

Curtis R. Pickering, Thomas J. Ow and Jeffrey N. Myers

Abstract Head and neck cancer is driven by the accumulation of genomic abnormalities. Classically these were characterized by chromosomal alterations and TP53 mutations, but recent sequencing studies have improved our understanding of the full spectrum of mutations in this disease. While a few oncogenes are found to be altered (PIK3CA, HRAS, CCND1), the most frequent events inactivate tumor suppressor genes (TP53, CDKN2A). A surprising new finding was the identification of mutations in the gene NOTCH1. These mutations demonstrate that NOTCH1 is an important tumor suppressor gene in head and neck and other squamous tumors. This has shed new light on the Notch pathway in head and neck cancer progression, and clarified our need to learn more about this pathway in the progression and biology of head and neck cancer.

Keywords Sequencing · Genomics · Notch · Head and neck cancer · Squamous

15.1 Early Findings in the Genomic Profiling of HNSCC

Initial studies examining the genomic alterations of head and neck squamous cell carcinoma (HNSCC) focused primarily on cytogenetic abnormalities and TP53 mutations [8]–[11], [22], [81]. Examination of large and medium-scale chromosomal alterations was experimentally feasible, while the technology at the time limited the ability to identify specific gene mutations in sporadic tumors. These early cytogenetic techniques included G-banding, fluorescence in situ hybridization (FISH), spectral

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karyotyping (SKY), comparative genomic hybridization (CGH), PCR-based allelotyping, and digital karyotyping. More recently, single nucleotide polymorphism (SNP) and molecular inversion probe (MIP) arrays have been utilized for cytogenetic analysis. These methods identify chromosomal regions with gains or losses relative to the normal copy number. It was clear from early studies that many chromosomal abnormalities were present in HNSCC [8], [22], [81], [38].

Initial chromosomal studies soon demonstrated that allelic loss on the short arm of chromosome 9 was a frequent event, as well as alterations on chromosomes 3, 8, 11, and 17 [14], [16], [50]. Deeper explorations of these highly altered regions led to gene-specific discoveries in HNSCC. The frequent deletions at chromosome locus 9p21 encompass the CDKN2A gene, which encodes the p16INK4A/p14ARF tumor suppressors [41], [62], [84], [90]. The frequent allelic loss on 17p was associated with TP53 mutation, as was found in other tumor types [1], [3], [9], [10], [11], [12], [13], [66]. TP53 mutations are extensively reviewed Chapter 12 (Roh and Koch). CDKN2A and TP53 are two of the most frequently altered genes in HNSCC, and are two of the most important tumor suppressor genes as well. Exploration of 11q, a frequently amplified region, identified CCND1, encoding cyclin D, as an important player in HNSCC [21], [26], [42], [44]. CCND1 amplification should promote proliferation, and antisense inhibition of cyclin D was shown to inhibit growth of HNSCC cell lines in vitro and in vivo [48], [74]. This region on 11q contains additional genes that may also be relevant to HNSCC progression including Fas-associated protein with death domain (FADD), an antiapoptotic gene, and ORAOV1 (overexpressed in oral cancer 1), so named based on its high expression in HNSCC. ORAOV1 has been shown to be necessary for HNSCC cell line growth [27]. Although ORAOV1 lacks known structural motifs the yeast homolog was recently found to regulate ribosome function and biogenesis in the presence of reactive oxygen species [88]. The copy number gains on chromosome 8 are usually linked to the well-described oncogene MYC [38].

Subsequent work examining a spectrum of oral lesions, ranging from dysplasia, to carcinoma in situ, to invasive carcinoma, demonstrated that these genetic aberrations appeared to occur in a progressive, albeit not a universally consistent, manner (Fig. 15.1) [14], [16], [40]. With this information, it was suggested that tumor development in HNSCC follows a stepwise model where a series of genetic events (perhaps 10 or more) occur before malignant transformation is complete, in a fashion similar to those models that were previously described for colorectal [73] and lung cancers [36].

15.1.1 "Next-gen" Sequencing

Recently, large-scale genome sequencing projects such as the Human Genome Project and The Cancer Genome Atlas (TCGA) have transformed our understanding of the human and cancer genome [37], [72]. They also launched a revolution in genome sequencing technology. A key consequence of these efforts was the development of "next-gen" sequencing methods [19], [43]. They are referred to as next-gen

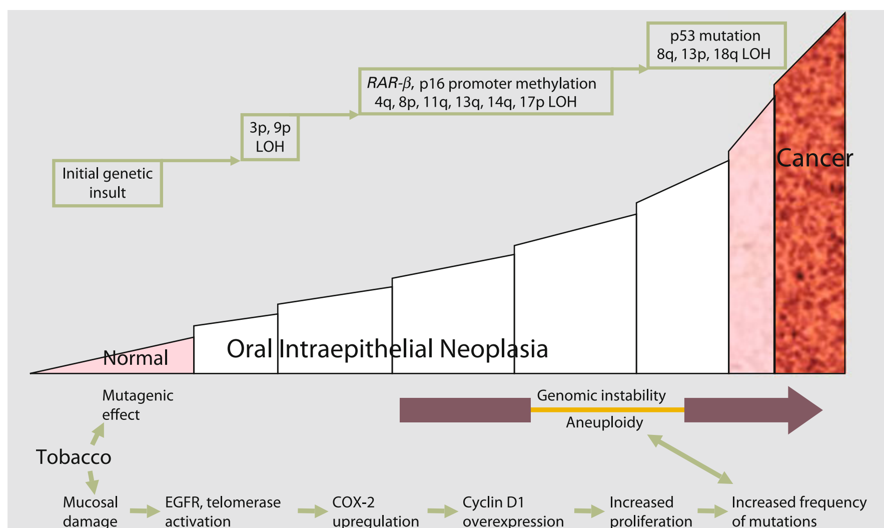

Fig. 15.1 Molecular (genetic and epigenetic) progression model of multistep oral carcinogenesis. The *white central steps* of the figure represent the progression of oral intraepithelial neoplasia from mild dysplasia through moderate and severe dysplasia or carcinoma in situ, which can precede cancer. This process involves activation of the epidermal growth factor receptor (*EGFR*) and related downstream events (e.g., involving cyclooxygenase-2 (*COX-2*) and cyclin D1) leading to dysregulated proliferation, increasing frequency of mutations causing genomic instability (and vice versa), and invasion. *LOH* loss of heterozygosity, *RAR-β* retinoic acid receptor-beta. (From [40], used with permission)

because the chemistry is different from the original Sanger sequencing methods. These refined sequencing techniques are orders of magnitude faster and more cost-effective than previous technology. These advances, paralleled by improvements in data storage and processing, have allowed for whole-genome and whole-exome sequencing projects. Specific details of next-gen technology and its many applications are beyond the scope of this chapter and have been reviewed elsewhere [19], [43]. Whole genome and exome sequencing are providing the most comprehensive picture to date of the alterations that occur during carcinogenesis. These exciting data demonstrate that the previously identified cytogenetic abnormalities and TP53 mutations only represent one piece of the genomic puzzle that is HNSCC.

Next-gen sequencing is already improving our understanding of HNSCC [2], [69]. The insight gained from the first two whole exome studies confirmed much of the existing knowledge about genetic events in HNSCC [2], [38], [69], but also provided new discoveries that have added to our understanding of these cancers. The remainder of this chapter discusses the results of next-generation sequencing in HNSCC with an expanded discussion of NOTCH mutations and the role of this pathway in HNSCC carcinogenesis.

15.2 Next-Generation Sequencing of HNSCC

In August 2011, the first whole-exome sequencing results in HNSCC were published by two groups [2], [69]. Stransky et al. [69] reported exome sequencing results on 74 patient samples with whole genome sequences for two patients, and Agrawal et al. [2] reported exome sequencing results from 32 patient samples and targeted sequencing of 8 genes in an additional 88 patients. Both groups also reported copy number analysis from SNP arrays. The differing methodological approaches in these studies (capture and sequencing platforms, mutation calling algorithms, validation protocols, etc.) prevent a direct comparison of many of the results. For example, Stransky et al. sequenced to 150-fold mean coverage while Agrawal et al. sequenced to 60-fold average coverage. Additionally, every mutation reported by Agrawal et al. was validated by a different sequencing chemistry (stringent threshold that will remove many events) while Stransky attempted to validate only 321 mutations and reported a validation rate of 89.7 % for those events (less stringent threshold that will remove fewer events). Because of these methodological differences, the average number of mutations per tumor is dramatically different between the studies (130 mutations/tumor in Stransky et al. vs. 19 mutations/tumor in Agrawal et al.). However, despite these differences, the most important findings about significant targets of mutation were corroborated by both groups, and some generalizations can be made across the studies.

Not surprisingly, more mutations were found in the tumors from patients with a history of tobacco use. This is consistent with the known function of tobacco smoke as a DNA-damaging agent and carcinogen. While neither study focused on the role of human papillomavirus (HPV) in HNSCC, both studies included a small number of patients identified as HPV positive (11 in Stransky et al. and 4 in Agrawal et al.) and both reported fewer mutations in those tumors. Since HPV-encoded proteins cause several carcinogenic disruptions in these tumors (e.g., E6 inhibits p53, E7 inhibits Rb), thus reducing the need for random mutagenesis to inactivate these genes, it is not surprising that HPV-positive tumors contained fewer total mutations. No mutations were observed in TP53 or CDKN2A in HPV-positive patients, indicating a lack of selective pressure for mutations in those pathways.

In general, the mutation spectrum of HNSCC shows a high level of diversity. TP53 was the only gene mutated in the majority of patients. All other altered genes were mutated in fewer than 15 % of patients, with most mutated in fewer than 10 %. The most commonly mutated genes identified in both studies included: TP53, NOTCH1, CDKN2A, PIK3CA, HRAS, CASP8, and TP63 (Fig. 15.2). Other genes found to be mutated in only one of the studies include: FAT1, PTEN, and FBXW7 (Fig. 15.2). Overall, the mutation spectrum was dominated by tumor suppressor genes. It was hoped that a frequently mutated oncogene could be identified and subsequently targeted for treatment. While this type of discovery occurred in GIST with c-KIT mutations and in melanoma with BRAF mutations, it is becoming clearer that these events are the exception rather than the rule. Most tumors have not been found to contain an easily identifiable oncogenic mutation, and this appears to be the case for HNSCC.

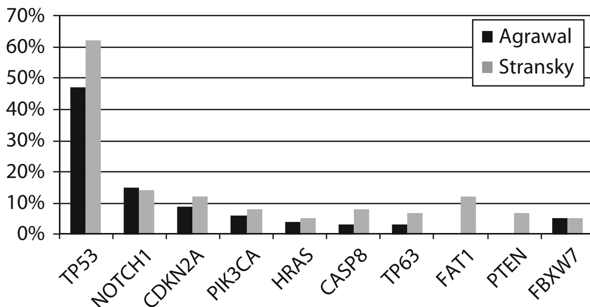

Fig. 15.2 Frequent mutations in HNSCC. (Mutation frequencies are taken from [2], [69])

15.2.1 TP53 (17p13.1)

TP53 is arguably the most frequently altered gene in human cancer, and it is well known that TP53 mutation is commonly identified in HNSCC, with rates ranging from 40–60 % in most studies [3], [9], [10], [11], [56], [57]. TP53 mutation and subclasses of mutations have been shown to have prognostic significance, as well [55], [57]. TP53 mutations were identified in 47 % of tumors overall, representing 78 % of HPV-negative tumors and no HPV-positive tumors, in the report by Agrawal et al. [2]. TP53 was altered in 62 % of tumors sequenced in the report by Stransky et al. [69]. Thus, whole-exome sequencing in HNSCC confirms the dominant role TP53 mutation plays in this disease, and the inverse relationship with HPV-related disease. Chapter 12 (Roh and Koch) discusses the biology of TP53 in HNSCC in great detail.

15.2.2 CDKN2A (9p21)

The CDKN2A genomic locus encodes two distinct tumor suppressor proteins, p16INK4A and p14ARF [64]. These two genes have distinct promoters and first exons, but share their second and third exons. The shared exons do not result in homologous proteins because they are translated in alternate reading frames. P16INK4A is a cyclin-dependent kinase inhibitor that blocks proliferation through the retinoblastoma (Rb) pathway by inhibiting CDK4/6. P14ARF functions in the p53 pathway by inhibiting the p53 ubiquitin ligase MDM2 and thus loss of P14ARF increases p53 levels. This locus is frequently inactivated during the carcinogenesis of many tumor types and can occur through deletion, DNA methylation, mutation or a combination of those events [64]. In humans, p16INK4A is generally thought to be the dominant target for these genomic events [64]. In HNSCC, p16INK4A is a very frequent target for inactivation [58], [62]. Methylation and deletion of p16INK4A in HNSCC have been reported by many groups [38], [62], while mutation had been reported less frequently [58]. Stransky et al. and Agrawal et al. found mutation of CDKN2A in 12 and 9 % of cases,

respectively. Many of these events were nonsense mutations, splice site mutations, or insertions or deletions resulting in a translational frameshift (frameshift indels) that are likely to inactivate or completely eliminate the protein. These events were generally more detrimental to p16INK4A than p14ARF, as had been seen previously [58], and support the role of p16INK4A as a dominant tumor suppressor gene in this tumor type. Both groups also identified copy number loss at this locus. With the emergence of more sensitive detection techniques, like next-gen sequencing, and multiplatform studies that examine all mechanisms of inactivation, like The Cancer Genome Atlas (TCGA) project, the estimation of the frequency of p16INK4A inactivation in HPV-negative HNSCC continues to rise and is likely to exceed 80 % in HPV-negative tumors. Conversely, HPV inactivates the Rb protein and removes the selective pressure for p16INK4A inactivation in HPV-positive HNSCC. Alterations at CDKN2A are generally not observed in HPV-positive tumors.

15.2.3 PIK3CA (3q26.3)/PTEN (10q23.3)

PIK3CA encodes the p110α catalytic subunit of the phosphatidylinositol 3-kinase (PI3K) enzyme [91]. PI3K is usually activated by growth factor signaling to generate phospholipid second messengers that regulate diverse cellular processes including: cellular growth, proliferation, survival, motility, and metabolism [91]. PIK3CA is a known oncogene that is mutated in many tumor types [91]. PIK3CA mutations were observed at approximately 7 % in the two recent studies in head and neck cancer, which is in accordance with the frequencies observed in previous reports [35], [59]. The most common mutations in PIK3CA, found in many tumor types, occur in the helical and kinase domains at amino acids E542, E545, and H1047 [5], [91] and result in increased lipid kinase activity and activation of downstream signaling pathways [25], [91]. These hotspot mutations were found in HNSCC. Noncanonical mutations are also found in HNSCC (K111, R115, Y343, T1052), but the functional impact of these mutations is unknown. PIK3CA copy number gains are also frequently observed in HNSCC [38]. It is unknown if copy number gains are biologically equivalent to activating mutations during the carcinogenic process.

Another class of genomic alteration found in this pathway is inactivating mutation of PTEN. PTEN is the phosphatase that removes the phosphorylation added by PI3K [91]. PTEN was mutated in 7 % of patients in Stransky et al., and these mutations included one splice site and one nonsense mutation. Mutations and deletions in PTEN in HNSCC have also been reported by others at frequencies approaching 10 % [53]. Activation of PI3K/PTEN signaling is one of the most obvious and frequent oncogenic events in this tumor type. Many therapeutics that target this pathway are currently in various stages of development [24], [83]. It is hoped that these drugs will prove beneficial in at least a subset of HNSCC patients, although it may be necessary to selectively treat patients with genomic alterations to this pathway. Additionally, it may be necessary to combine PI3K/PTEN pathway inhibitors with additional drugs that target compensatory pathways [86].

15.2.4 HRAS (11p15.5)

The RAS genes are members of a family of GTPases involved in growth factor signaling. These genes can be potent oncogenes that activate both PI3K/PTEN signaling and MAPK pathway signaling [85]. The three primary RAS family members in humans are HRAS, KRAS, and NRAS. Each of these genes has been found to be mutated in cancer [5], [85]. HRAS is the only RAS family member frequently mutated in HNSCC (approximately 5 %) [2], [69]. These mutations occur in the known hotspot regions (G12, G13, Q61) [5] and have been shown to activate HRAS signaling. RAS activation has proven extremely difficult to selectively target with small molecule therapeutics [85], and RAS status is primarily used to identify resistance to therapeutics in other tumor types. KRAS mutations predict resistance to epidermal growth factor receptor (EGFR) targeted therapeutics in lung [15] and colorectal cancer [7], and it is likely that HRAS mutations will have a similar role in HNSCC. While HRAS mutations may not be directly targetable it still hoped that they will become clinically useful in HNSCC in terms of predicting outcome, guiding treatment selection, or insensitivity to drugs targeting downstream effectors. It is interesting that the spectrum of RAS family member mutations is different across tumor types. For example, KRAS is the primary target for RAS mutations in lung and colorectal cancer, but HRAS is the primary target in HNSCC [2], [69]. The biological reason for this difference is unknown.

15.2.5 EGFR (7p12)

The EGFR is the target for oncogenic activation in many tumor types through either copy number amplifications or mutation [7], [15]. These events promote tumor cell proliferation. In HNSCC, EGFR is a frequent target for gene amplification [38]. The EGFRvIII mutation is an in-frame deletion of exons 2–7 that is associated with poor prognosis and lack of response to EGFR inhibitory antibodies in gliomas. While EGFRvIII mutations have been reported in HNSCC at very high frequencies [65], neither of the recent studies identified any canonical EGFR mutations, including EGFRvIII. Hence, although EGFR is an important growth factor in HNSCC, it does not appear to be a frequent target for mutation.

15.2.6 CASP8 (2q23-q34)

Caspase-8 (CASP8) is a key intermediate in the extrinsic apoptotic signaling pathway [71], [76]. Although caspases are integral to apoptotic signaling and tumors often disrupt apoptotic signaling pathways, caspases are not frequently mutated in tumors [5], [30], [68]. Both of the recent papers identified CASP8 mutations at a low frequency (8 and 3 %). Most of these mutations are nonsense and thus predicted to

disrupt function of the protein. So far, colorectal adenocarcinoma is the only other tumor type with frequent CASP8 mutations [30], [51]. The most significant insight into the role of CASP8 mutations in HNSCC comes from a mouse model in which CASP8 is knocked out in the skin [34]. These mice develop a hyperproliferative and inflammatory phenotype in the skin. This suggests that CASP8 loss may not be sufficient for tumor formation, but its loss may contribute to carcinogenesis. Additionally, many HNSCC are highly infiltrated by immune cells; it is possible that disruption of CASP8 plays a role in this phenotype in some tumors.

15.2.7 FAT1 (4q35)

FAT1 is a large transmembrane protein that is a member of the cadherin family [67], [70]. FAT1 was mutated in 12 % of patients in the Stransky et al. paper. The majority of these mutations were nonsense. Additionally, FAT1 deletions have been found in HNSCC [49]. These data along with very limited in vitro studies suggest that FAT1 may be a tumor suppressor in HNSCC. Based on homology with the Drosophila FAT genes, FAT1 may regulate E-cadherin or the Hippo pathway [67], [70]. A recent study also demonstrated that FAT1 can regulate β-catenin and wnt signaling [47]. FAT1 is one of the most exciting new candidate tumor suppressor genes identified by next-gen sequencing.

15.2.8 FBXW7 (4q31.3)

FBXW7 is an F-box protein and part of the SCF (SKP1, CUL1, F-box) ubiquitin ligase complex [77], and controls the cell cycle regulated degradation of many proteins. FBXW7 has three hotspot sites for mutation (R465, R479, R505) [4], [5]. These residues are necessary for substrate binding, which is abrogated by mutations at these sites, thus inactivating FBXW7 function [77]. FBXW7 was mutated in 5 % of patients in both recent papers, and some of these mutations occurred in the known hotspots. Targets of FBXW7 include known oncogenes such as; MYC, Cyclin E, Jun, and Notch [77], but the targets for FBXW7 in HNSCC are still unknown. The possible role of FBXW7 mutations in regulating Notch signaling in HNSCC will be discussed further below.

15.2.9 TP63 (3q28)

TP63 is a p53 family member. TP63 is homologous to TP53 but it also has many unique functions [78], the most important of which is its role in epithelial squamous differentiation [33]. This unique role is dramatically demonstrated by the TP63

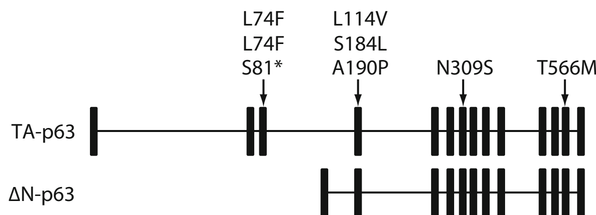

Fig. 15.3 TP63 mutations and splice isoforms. The exon structure for the TP63 N-terminal isoforms is shown. TA-p63 utilizes three unique N-terminal exons while DN-p63 utilizes an additional unique exon. Three of the observed TP63 mutations occurred in exons unique to the TA-p63 isoform. While there are also three C-terminal isoforms, this diagram represents only the alpha C-terminal isoform. * indicates a nonsense mutation. (Mutations are taken from [2], [69] and Pickering et al. unpublished data)

knockout mouse model, as neonatal mice die of dehydration shortly after birth due to a lack of skin [45], [82]. P63 maintains epithelial stem cells and the levels of p63 drop during epithelial squamous differentiation [33]. In this way, it is easy to understand how p63 can function as an oncogene during HNSCC. In support of this, TP63 has copy number gains in approximately 50 % of HNSCC [38]. While this appears very simple, the biology of TP63 in HNSCC has an additional layer of complexity caused by multiple splice variants. There are two major splice forms of p63 referred to as TA-p63 and *Δ*N-p63. The TA-p63 contains the N-terminal transactivation domain (TA) while the *Δ*N-p63 utilizes an alternate first exon and lacks the N-terminal TA domain. There are also C-terminal splice variants, and a full discussion of those forms can be found elsewhere [78]. It has been shown that *Δ*N-p63 is the predominant isoform in HNSCC and this form acts as an oncogene, while TA-p63 acts as a tumor suppressor and can inhibit HNSCC cell line proliferation, migration, invasion, and survival [46], [63]. TP63 mutations were found in about 5 % of patients in both recent sequencing studies. Many of these mutations occur in exons exclusive to the TA-p63 isoform (Fig. 15.3). Based on the biology of p63 it is likely that these mutations function to inhibit the tumor suppressive functions of the TA-p63 isoform.

15.3 The Emergence of NOTCH Signaling

One of the most exciting discoveries from next-gen sequencing of HNSCC was the identification of inactivating mutations in NOTCH1 (9q34.3) that indicated it was a tumor suppressor gene. Notch was originally discovered through analysis of a strain of Drosophila with notched wings [29], [79]. The mutated gene causing this phenotype was found to regulate patterning and morphogenesis in flies, signaling through the Enhancer of split complex, and in coordination with Delta, Numb, wingless, Inscuteable, and Prospero. Notch is also an important gene in mammals with a role in cell–cell signaling and differentiation, particularly epithelial squamous differentiation [20], [32], [60].

15.3.1 Notch in Skin Differentiation and Carcinogenesis

The major function of Notch in HNSCC is likely to be related to its role in normal squamous epithelium. Notch is an important transcription factor for epithelial squamous differentiation. During normal squamous differentiation the levels of NOTCH1 are low in the basal layer and progressively increase across the differentiating epithelium [20], [60], while p63 shows the opposite pattern. Notch1 and p63 have an antagonistic signaling relationship [20] that regulates many genes involved in squamous differentiation including: p21, involucrin, keratin 1 and keratin 10 [20], [60]. Since both Notch1 and p63 are frequent targets for genomic alteration in HNSCC it is likely that this common pathway is important for tumorigenesis. The observed genomic alterations generally activate p63 and inhibit Notch1. It is likely that alterations in p63 and Notch1 will be mutually exclusive in HNSCC, but so far no dataset has been able to test this hypothesis. Based on information from the skin model systems, these events should promote a less differentiated state in tumor cells. Interestingly, most HNSCC specimens exhibit some level of squamous differentiation, often including round bundles of keratin (keratin pearls). This is even commonly observed in tumors graded as poorly differentiated, and it suggests that alteration of differentiation pathways may not result in a complete block to terminal differentiation; instead it may promote a less differentiated state that is beneficial to the tumor. Reactivation of terminal differentiation pathways could be a viable therapeutic strategy, if the appropriate target(s) could be identified.

The NOTCH1 knockout mouse is embryonic lethal. However, a skin-specific (keratin 5-proter driven) Notch knockout is viable and develops a dermal hyperplasia [19], [52], [61]. This hyperplasia includes increased proliferation, expansion of the basal layer, and altered expression of differentiation markers, consistent with the role of Notch1 in differentiation. These mice also develop spontaneous skin tumors and are at increased susceptibility for formation of chemically induced tumors (DMBA-TPA protocol) [52]. This demonstrated that Notch1 is a tumor suppressor gene in mouse skin, and the human mutation data confirm that this model is likely to be consistent in human SCC tumors. The Notch pathway in skin carcinogenesis is likely to interact with many other pathways. Activated v-Ha-ras selectively promoted tumorigenesis in Notch1 knockout keratinocytes [52], and signaling interactions with EGFR, p53, and NFκB have been shown [20].

15.3.2 Notch as a Tumor Suppressor or Oncogene

Notch signaling is disrupted in many tumor types, and these disruptions can either activate or inhibit Notch signaling. Notch can function as either an oncogene or a tumor suppressor gene, depending on the tumor type that is studied [60]. Pediatric acute lymphoblastic leukemia (ALL) exemplifies this dual role for Notch signaling. During lymphocyte differentiation a precursor cell can differentiate to assume either a T-cell or a B-cell lineage. Notch controls this lineage specification [92]. Activation

of Notch signaling promotes the T-cell lineage, while inhibition of Notch signaling promotes the B-cell lineage. ALL with a T-cell differentiation (T-ALL) very frequently contains genomic alterations that activate Notch signaling. These tumors can have activating mutations in Notch1, or a translocation, t(7; 9) (q34; q34.3), that leads to overexpression of a Notch1 protein that resembles NICD [92]. Activation of Notch is a nearly universal event in T-ALL. In contrast, in ALL a B-cell differentiation (B-ALL) Notch signaling is detrimental to the cells [93]. In vitro activation of Notch signaling results in growth arrest and apoptosis of B-ALL cells [92]. Frequent genomic alterations that block Notch signaling in B-ALL have not yet been identified. Altered Notch signaling has now been identified in multiple hematopoietic and solid tumor types (5). Next-gen sequencing has facilitated the discovery of genomic alterations in Notch or its regulators in many of these tumors.

15.3.3 Notch Signaling

There are four Notch genes in humans (NOTCH1–4). Each of these genes encodes a large transmembrane receptor controlled by multiple levels of regulation. Some of the key domains in Notch are: multiple N-terminal extracellular EGF-like domains where ligand binding occurs, an extracellular heterodimerization domain, a transmembrane domain, and a C-terminal PEST (P-proline, E-glutamic acid, S-serine, T-threonine rich) domain that is a signal for degradation [32]. The first level of regulation occurs during processing in the Golgi apparatus where O-glycosylation is added to the EGF domains [32]. These modifications can influence ligand binding. The next level of regulation is by furin convertase which cleaves the Notch protein between the extracellular and transmembrane domains [32]. This cleavage converts the single Notch protein into a noncovalently bound heterodimer that is anchored in the cell membrane. This processed form of Notch is then regulated by ligand binding.

The ligands for Notch are members of the Delta (DLL1, DLL3, DLL4) and Jagged (JAG1, JAG2) families [32]. These proteins are usually found in the membrane of an adjacent cell and interact with the EGF repeats in the Notch extracellular domain. Additional specificity is gained by having multiple Notch proteins and multiple ligands. While the various combinations of receptor and ligand have different levels of signaling activity in various model systems [87], the physiological result of this complexity is not well understood. Ligand binding leads to additional Notch cleavage events. The first cleavage is by ADAM (a disintegrin and metalloprotease) proteases on the N-terminal region of the transmembrane subunit of Notch. ADAM cleavage primes Notch for final cleavage by γ-secretase within the transmembrane domain. This cleavage releases the active, functional form of Notch into the cytoplasm: this active form is usually referred to as the Notch intracellular domain (NICD) [32]. The NICD translocates to the nucleus and, along with coactivators (RBPjk and MAML1–3) activates transcription of the HES and HEY genes [32]. Finally, at the end of activation, NICD is phosphorylated by CDK8 in its C-terminal PEST domain. This signals NICD for degradation by the E3 ubiquitin ligase FBXW7 [77]. As Notch is usually a short-lived protein, this allows for very controlled regulation of Notch signaling.

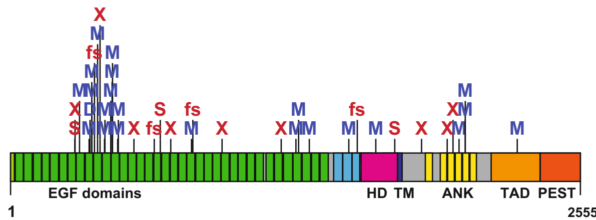

Fig. 15.4 NOTCH1 mutations observed in HNSCC. A cluster of mutations are observed in the *EGF* domains, indicating a tumor suppressor function. *M* missense mutation, *D* in-frame deletion, *X* nonsense mutation, *S* splice site mutation, *fs* frame shift insertion or deletion, *HD* heterodimerization domain, *TM* transmembrane domain, *ANK* ankyrin domains, *TAD* transcriptional activation domain. (Mutations are taken from [2], [69], and Pickering et al. unpublished data)

15.3.4 Genomic Alterations in Notch Signaling

NOTCH1 is the most frequent target in the Notch pathway for genomic alterations, with mutations the most frequent alteration observed. Over 1,000 unique samples with NOTCH1 mutations are noted in the COSMIC mutation database [5]. These mutations are clustered in two regions of the protein: the extracellular heterodimerization domain and the C-terminal PEST domain (Fig. 15.4). Missense mutations and insertions and deletions (indels) are observed in both major regions. Many of the indels are predicted to result in a translational frameshift. Mutations in the heterodimerization domain promote cleavage and activation of Notch1 without the need for ligand binding. Mutations near the PEST domain also promote Notch signaling by increasing the stability of the NICD fragment. Many of the mutations in this region prematurely truncate the protein (either through a nonsense or frameshift mutation) and thus eliminate the PEST domain. These most common mutations are generally found in T-cell leukemias and are considered oncogenic [60].

While mutation of NOTCH1 can eliminate FBXW7 binding, mutation of FBXW7 can also block Notch1 binding. As noted above, there are three mutation hotspots within FBXW7 that all lead to a reduced ability to bind substrates. These mutations are frequently observed in T-ALL (30 %) and less frequently in many other tumor types (6 %) [77]. This is another mechanism to stabilize the Notch1 protein. FBXW7 has other targets aside from Notch1, including MYC and γ-secretase, which are in the Notch pathway. It is possible that FBXW7 activates the Notch pathway at multiple levels by stabilizing γ-secretase (an activator of Notch1) and MYC (a Notch1 target gene). Clearly, FBXW7 mutation has oncogenic consequences beyond Notch signaling.

Other mutations in the Notch pathway have been identified by next-gen sequencing in chronic myelomonocytic leukemia (CMML) [31]. Mutations were reported in Nicastrin, APH1A, MAML1, and NOTCH2. These genes encode proteins situated at various stages of the Notch signaling pathway, with Nicastrin and APH1A part of the γ-secretase complex and MAML1 part of the NICD transcription complex. These mutations are believed to inhibit Notch signaling and suggest a tumor suppressive role

for Notch in this disease [31]. It is possible that other members of the Notch pathway are also mutated in HNSCC and contribute to pathway dysfunction. However, the frequency of these events is likely to be low.

For reasons of simplicity, we have not so far discussed the role of other members of the Notch family. Overall, Notch1 is the primary target for mutations in cancer, and most of our understanding of the pathway is based on work with Notch1. However, some studies have evaluated the role of other Notch family members, and generally the ligand specificity and activation of downstream targets can vary somewhat from that of Notch1 [87]. In serous ovarian cancer NOTCH3 is amplified and overexpressed [54]. This is an oncogenic event and the consequences are expected to be similar to those that occur with Notch1 activation.

15.3.5 Notch Mutations in HNSCC and Other Squamous Tumors

NOTCH1 mutations were found in 14 and 15 % of cases in Stransky et al. and Agrawal et al., respectively (Fig. 15.4). This finding was made even more exciting because the spectrum of mutations was dramatically different from that of T-ALL. Many of the mutations were nonsense or frameshift and predicted to inactivate the protein. Additionally, the mutations were not clustered in the heterodimerization domain or near the PEST domain. Instead, they were spread across the protein with some clustering in the EGF repeats near the ligand-binding region. This spectrum of mutations provided very strong evidence that NOTCH1 was acting as a tumor suppressor in HNSCC, making this the first demonstrations of tumor suppressive Notch mutations in a solid tumor. Similar NOTCH1 mutations were later reported in cutaneous and lung squamous cell carcinoma (SCC) [75]. Two of the mutations from cutaneous SCC that occurred in the ligand-binding region were found to prevent activation of Notch signaling by the ligand in an in vitro assay [75]. This confirmed the predicted inhibitory nature of the NOTCH1 mutations.

Mutations were also identified in the Notch family members, NOTCH2, 3, and 4 at lower frequencies in head and neck cancer [2], [69], [75]. The impact of these mutations is still unknown. Since the frequency of mutation is lower in these genes, there have been relatively few mutations observed. Therefore, it is difficult to discern a pattern among these events, and it is still unclear if the spectrum of mutations is similar to those observed in NOTCH1.

Mutations observed in cancer can be thought of as driver or passenger mutations. Driver mutations are beneficial to the tumor cells and are selected during the evolution of the tumor. A high frequency of mutation within a population of patients is a good indicator that a gene might be a driver. The other possibility is that the mutation occurred randomly but was an evolutionary passenger with other beneficial mutations. For example, random gene X might be mutated in the same cells that acquired a TP53 mutation. Selection for the TP53 mutation would also result in selection for the mutation in random gene X. The probability of finding a mutation in random gene X is related to the background mutation rate in that gene.

Tumors with high background mutation rate, often caused by carcinogen exposure or dysfunction in DNA repair genes, will have lots of passenger mutations but not necessarily more driver mutations. Statistical models have been developed to help discern driver mutations from passengers [6]. Mutations in NOTCH2, 3, and 4 have not yet reached statistical significance as driver genes in HNSCC using these models. This does not, however, mean that they are not relevant to the biology of HNSCC. Rare events can still be biologically important even if they do not occur at a high frequency. In the case of a known oncogene and a known hotspot mutation, it is easy to make a biological argument for the relevance of that mutation to the tumor. In light of these concepts, it is at present challenging to make a strong biological argument for the importance of NOTCH2, 3, and 4 mutations. This model would require that all Notch family members be necessary for signaling through the pathway, so that loss of any individual member blocks the pathway. Similarly, it requires that Notch family members do not compensate for each other. Another biological argument is that different subtypes of HNSCC each require a different Notch family member for signaling. This model predicts that NOTCH1 mutant tumors would be different in some way from NOTCH2 mutant tumors. This has not yet been observed and will require a large sample set, such as the upcoming TCGA project, for validation. It will be important to further characterize the mutations in Notch family members for their impact on HNSCC.

15.3.6 The Future of Notch in HNSCC

The identification of frequent genomic alterations in NOTCH1 in HNSCC demonstrated that this gene is likely to be important for HNSCC carcinogenesis. In vitro and in vivo data have confirmed that NOTCH1 is a significant tumor suppressor in HNSCC and other squamous tumors [60]. However, there are still many critical unknowns in our understanding of Notch in HNSCC. For example, the clinical impact of NOTCH1 mutations has not yet been described. It is possible that NOTCH1 mutations will be associated with poorly differentiated tumors and worse outcome, based on our understanding of Notch biology. Large, well-annotated clinical cohorts must be collected and analyzed for NOTCH1 mutations, differentiation state, and outcome. NOTCH1 may also have predictive significance for cisplatin or radiation treatment. It will be necessary to identify cohorts to address these questions as well.

Our understanding of Notch signaling must also be improved. Currently there is not an effective biomarker for the presence of a functional Notch pathway. It is likely that NOTCH1 mutations are not the only genomic alteration that disrupts this pathway. Copy number loss of other key members of the pathway may also prevent activation. MAML1 is part of the Notch transcriptional complex and this gene is located in chromosome 5q35, which is frequently lost in HNSCC [38]. It is possible that loss of 5q reduces Notch signaling, but this must be examined experimentally. Similarly, it is unknown if mutation or amplification of TP63 has the same functional impact on Notch signaling as NOTCH1 mutation. It will be

important to develop markers for activation and inhibition of the Notch pathway that can be utilized in vitro and in vivo. A good biomarker for the pathway will aid in the identification of additional genomic alterations that contribute to Notch pathway dysfunction. This will facilitate a determination of the frequency of Notch pathway inactivation. NOTCH1 mutations occur in 15 % of tumors and TP63 is mutated in 6 % and amplified in as many as 50 % of tumors. It is possible that Notch pathway inactivation occurs in more than 50 % of HNSCC.

Usually, Notch requires ligand binding for activation. The source of Notch ligand in HNSCC is not known. The likely possibilities for providing ligand include other tumor cells, stromal cells or immune cells. The source of the ligand has important implications for Notch biology and targetability in HNSCC, and it could indicate why only a subset of tumors mutates NOTCH1. For example, if the ligand were expressed on immune cells then only tumors with immune infiltration would be expected to mutate NOTCH1. In this case, it might be possible to use an artificial ligand/agonist of Notch as a treatment in tumors without immune infiltration. It is also possible that the tumor cells are the source of ligand and the cells express both the ligand and receptor. This scenario also has a treatment implication. High expression of the ligand on the same cell as the receptor can block signaling through a process called cis-inhibition [17]. It is possible that modest levels of ligand activate Notch, while high levels of ligand become a resistance mechanism and block the pathway. In this case, it may be possible to disrupt the cis-inhibition and lead to activation of the pathway. Many details of Notch signaling in HNSCC must be further characterized both in vitro and in vivo.

HPV influences the biology and genomics of a subset of HNSCC. While there is currently limited genomic data on HPV-positive tumors, it appears that the mutational profile is different. Interestingly, NOTCH1 mutations were still observed in HPV-positive tumors. This is especially surprising since the HPV E6 protein has been shown to bind MAML1 and possibly alter its function [80]. It will be important to investigate the role of Notch signaling in HPV-positive disease and determine if the biology of Notch is altered by the presence of HPV.

Notch clearly has a dual role in human cancer. It is an oncogene in some tumor types and a tumor suppressor in others. In HNSCC Notch appears to be a tumor suppressor. However, it is possible that in a subset of HNSCC Notch is acting as an oncogene. Early reports of immunohistochemical staining in HNSCC (with antibodies of questionable specificity) concluded that many cases had high levels of activated Notch [39], [28], [23], [90]. There is also one report where overexpression of NICD in a HNSCC cell line promoted proliferation [39]. The genomic data do not yet rule out this possibility, since the functional consequences of most NOTCH1 mutations have not yet been examined in vitro. It is possible that a subset of the mutations activate Notch signaling. If Notch is oncogenic in a subset of HNSCC then it will be necessary to understand the biological and clinical implications of that finding. It will be important to eliminate the possibility of oncogenic Notch, if treatment is eventually tailored to the Notch status of the tumor.

15.4 Conclusions

Next-gen sequencing is a powerful research tool that is dramatically improving our understanding of the biology of HNSCC. It has validated many of the known genomic alterations while identifying a few novel events, particularly the discovery of frequent inactivating mutations in NOTCH1. These events solidify NOTCH1 as an important tumor suppressor pathway in HNSCC and make it imperative that more is learned about the details of Notch signaling in this disease. There are still many questions regarding the biology and clinical consequences of Notch pathway disruption that will require follow-up studies, both in vitro and in vivo. It will also be important to understand the role of Notch signaling in the context of other genes known to be important in HNSCC.

Finally, our increased understanding of HNSCC has not yet led to new effective therapies. While genomics has improved our understanding of this disease it has not identified biomarkers that dictate treatment strategies, or oncogenes amenable to targeted therapy. HNSCC is dominated by the loss of several tumor suppressor genes, and much more work must be done to develop strategies to use genomic information to battle this disease.

References

1. Adamson R, Jones AS, Field JK. Loss of heterozygosity studies on chromosome 17 in head and neck cancer using microsatellite markers. Oncogene. 1994;9(7):2077–82.
2. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333 (6046):1154–7. doi: 10.1126/science.1206923.
3. Ahomadegbe JC, Barrois M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P, Armand JP, Riou G. High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene. 1995;10(6):1217–27.
4. Akhoondi S, Sun D, der Lehr N von, Apostolidou S, Klotz K, Maljukova A, Cepeda D, Fiegl H, et al. FBXW7/hCDC4 is a general tumor suppressor in human cancer. Cancer Res. 2007;67(19):9006–12. doi: 10.1158/0008-5472.CAN-07-1320.
5. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br J Cancer. 2004;91(2):355–8. doi: 10.1038/sj.bjc.6601894.
6. Banerji S, Cibulskis K, Rangel-Escareno C, Brown KK, Carter SL, Frederick AM, Lawrence MS, Sivachenko AY, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. Nature. 2012;486(7403):405–9. doi: 10.1038/nature11154.
7. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol: Off J Am Soc Clin Oncol. 2010;28(7):1254–61. doi: 10.1200/JCO.2009.24.6116.
8. Bockmuhl U, Petersen I. DNA ploidy and chromosomal alterations in head and neck squamous cell carcinoma. Virchows Arch: Int J Pathol. 2002;441(6):541–50. doi: 10.1007/s00428-002-0729-3.

9. Boyle JO, Hakim J, Koch W, Riet P van der, Hruban RH, Roa RA, Correo R, Eby YJ, et al. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res. 1993;53(19):4477–80.
10. Brennan JA, Boyle JO, Koch WM, Goodman SN, Hruban RH, Eby YJ, Couch MJ, Forastiere AA, et al. Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. New Engl J Med. 1995;332(11):712–7. doi: 10.1056/NEJM199503163321104.
11. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. New Engl J Med. 1995;332(7):429–35. doi: 10.1056/NEJM199502163320704.
12. Burns JE, Baird MC, Clark LJ, Burns PA, Edington K, Chapman C, Mitchell R, Robertson G, et al. Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. Br J Cancer. 1993;67(6):1274–84.
13. Caamano J, Zhang SY, Rosvold EA, Bauer B, Klein-Szanto AJ. p53 alterations in human squamous cell carcinomas and carcinoma cell lines. Am J Pathol. 1993;142(4):1131–9.
14. Califano J, Riet P van der, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res. 1996;56(11):2488–92.
15. Carter CA, Giaccone G. Treatment of nonsmall cell lung cancer: overcoming the resistance to epidermal growth factor receptor inhibitors. Curr Opin Oncol. 2012;24(2):123–9. doi: 10.1097/CCO.0b013e32834ec6a7.
16. Choi S, Myers JN. Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. J Dent Res. 2008;87(1):14–32.
17. del Alamo D, Rouault H, Schweisguth F. Mechanism and significance of cis-inhibition in Notch signalling. Curr Biol: CB. 2011;21(1):R40–7. doi: 10.1016/j.cub.2010.10.034.
18. Demehri S, Turkoz A, Kopan R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. Cancer cell. 2009;16(1):55–66. doi: 10.1016/j.ccr.2009.05.016.
19. Ding L, Wendl MC, Koboldt DC, Mardis ER. Analysis of next-generation genomic data in cancer: accomplishments and challenges. Hum Mol Genet. 2010;19(R2):R188–96. doi: 10.1093/hmg/ddq391.
20. Dotto GP. Crosstalk of Notch with p53 and p63 in cancer growth control. Nature Rev Cancer. 2009;9(8):587–95. doi: 10.1038/nrc2675.
21. Gaffey MJ, Iezzoni JC, Meredith SD, Boyd JC, Stoler MH, Weiss LM, Zukerberg LR, Levine PA, et al. Cyclin D1 (PRAD1, CCND1) and glutathione-S-transferase pi gene expression in head and neck squamous cell carcinoma. Hum Pathol. 1995;26(11):1221–6.
22. Gollin SM. Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. Head Neck. 2001;23(3):238–53.
23. Hijioka H, Setoguchi T, Miyawaki A, Gao H, Ishida T, Komiya S, Nakamura N. Upregulation of Notch pathway molecules in oral squamous cell carcinoma. Int J Oncol. 2010;36(4):817–22.
24. Ihle NT, Powis G. Take your PIK: phosphatidylinositol 3-kinase inhibitors race through the clinic and toward cancer therapy. Mol Cancer Ther. 2009;8(1):1–9. doi: 10.1158/1535-7163.MCT-08-0801.
25. Ikenoue T, Kanai F, Hikiba Y, Obata T, Tanaka Y, Imamura J, Ohta M, Jazag A, et al. Functional analysis of PIK3CA gene mutations in human colorectal cancer. Cancer Res. 2005;65(11):4562–7. doi: 10.1158/0008-5472.CAN-04-4114.
26. Jares P, Fernandez PL, Campo E, Nadal A, Bosch F, Aiza G, Nayach I, Traserra J, et al. PRAD-1/cyclin D1 gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas. Cancer Res. 1994;54(17):4813–7.
27. Jiang L, Zeng X, Yang H, Wang Z, Shen J, Bai J, Zhang Y, Gao F, et al. Oral cancer overexpressed 1 (ORAOV1): a regulator for the cell growth and tumor angiogenesis in oral squamous cell carcinoma. Int J Cancer (Journal international du cancer). 2008;123(8):1779–86. doi: 10.1002/ijc.23734.

28. Joo YH, Jung CK, Kim MS, Sun DI. Relationship between vascular endothelial growth factor and Notch1 expression and lymphatic metastasis in tongue cancer. Otolaryng Head Neck Surg: Off J Am Acad Otolaryng Head Neck Surg. 2009;140(4):512–8. doi: 10.1016/j.otohns.2008.12.057.
29. Kidd S, Kelley MR, Young MW. Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors. Mol Cell Biol. 1986;6(9):3094–108.
30. Kim HS, Lee JW, Soung YH, Park WS, Kim SY, Lee JH, Park JY, Cho YG, et al. Inactivating mutations of caspase-8 gene in colorectal carcinomas. Gastroenterology. 2003;125(3):708–15.
31. Klinakis A, Lobry C, Abdel-Wahab O, Oh P, Haeno H, Buonamici S, De Walle I van, Cathelin S, et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature. 2011;473(7346):230–3. doi: 10.1038/nature09999.
32. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137(2):216–33. doi: 10.1016/j.cell.2009.03.045.
33. Koster MI, Dai D, Roop DR. Conflicting roles for p63 in skin development and carcinogenesis. Cell cycle. 2007;6(3):269–73.
34. Kovalenko A, Kim JC, Kang TB, Rajput A, Bogdanov K, Dittrich-Breiholz O, Kracht M, Brenner O, et al. Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J Exp Med. 2009;206(10):2161–77. doi: 10.1084/jem.20090616.
35. Kozaki K, Imoto I, Pimkhaokham A, Hasegawa S, Tsuda H, Omura K, Inazawa J. PIK3CA mutation is an oncogenic aberration at advanced stages of oral squamous cell carcinoma. Cancer Sci. 2006;97(12):1351–8. doi: 10.1111/j.1349-7006.2006.00343.x.
36. Kratzke RA, Shimizu E, Kaye FJ. Oncogenes in human lung cancer. Cancer Treat Res. 1992;63:61–85.
37. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860–921. doi: 10.1038/35057062.
38. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nature Rev. Cancer. 2011;11(1):9–22. doi: 10.1038/nrc2982.
39. Lin JT, Chen MK, Yeh KT, Chang CS, Chang TH, Lin CY, Wu YC, Su BW, et al. Association of high levels of Jagged-1 and Notch-1 expression with poor prognosis in head and neck cancer. Ann Surg Oncol. 2010;17(11):2976–83. doi: 10.1245/s10434-010-1118-9.
40. Lippman SM, Sudbo J, Hong WK. Oral cancer prevention and the evolution of molecular-targeted drug development. J Clin Oncol: Off J Am Soc Clin Oncol. 2005;23(2):346–56. doi: 10.1200/JCO.2005.09.128.
41. Lydiatt WM, Murty VV, Davidson BJ, Xu L, Dyomina K, Sacks PG, Schantz SP, Chaganti RS. Homozygous deletions and loss of expression of the CDKN2 gene occur frequently in head and neck squamous cell carcinoma cell lines but infrequently in primary tumors. Genes Chromosomes Cancer. 1995;13(2):94–8.
42. Meredith SD, Levine PA, Burns JA, Gaffey MJ, Boyd JC, Weiss LM, Erickson NL, Williams ME. Chromosome 11q13 amplification in head and neck squamous cell carcinoma. Association with poor prognosis. Arch Otolaryngol Head Neck Surg. 1995;121(7):790–4.
43. Meyerson M, Gabriel S, Getz G. Advances in understanding cancer genomes through second-generation sequencing. Nature Rev Genetics. 2010;11(10):685–96. doi: 10.1038/nrg2841.
44. Michalides R, Veelen N van, Hart A, Loftus B, Wientjens E, Balm A. Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. Cancer Res. 1995;55(5):975–8.
45. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999;398(6729):708–13. doi: 10.1038/19531.
46. Mitani Y, Li J, Weber RS, Lippman SL, Flores ER, Caulin C, El-Naggar AK. Expression and regulation of the DeltaN and TAp63 isoforms in salivary gland tumorigenesis clinical and experimental findings. Am J Pathol. 2011;179(1):391–9. doi: 10.1016/j.ajpath.2011.03.037.
47. Morris LG, Kaufman AM, Gong Y, Ramaswami D, Walsh LA, Turcan S, Eng S, Kannan K, et al. Recurrent somatic mutation of FAT1 in multiple human cancers leads to aberrant Wnt activation. Nat Genet. 2013;45(3):253–61. doi: 10.1038/ng.2538.

48. Nakashima T, Clayman GL. Antisense inhibition of cyclin D1 in human head and neck squamous cell carcinoma. Arch Otolaryngol-head Neck Surg. 2000;126(8):957–61.
49. Nakaya K, Yamagata HD, Arita N, Nakashiro KI, Nose M, Miki T, Hamakawa H. Identification of homozygous deletions of tumor suppressor gene FAT in oral cancer using CGH-array. Oncogene. 2007;26(36):5300–8. doi: 10.1038/sj.onc.1210330.
50. Nawroz H, Riet P van der, Hruban RH, Koch W, Ruppert JM, Sidransky D. Allelotype of head and neck squamous cell carcinoma. Cancer Res. 1994;54(5):1152–5.
51. Network, The Cancer Genome Atlas. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330–7. doi: 10.1038/nature11252.
52. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, Noort M van, Hui CC, Clevers H, et al. Notch1 functions as a tumor suppressor in mouse skin. Nature Genet. 2003;33(3):416–21. doi: 10.1038/ng1099.
53. Okami K, Wu L, Riggins G, Cairns P, Goggins M, Evron E, Halachmi N, Ahrendt SA, et al. Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors. Cancer Res. 1998;58(3):509–11.
54. Park JT, Li M, Nakayama K, Mao TL, Davidson B, Zhang Z, Kurman RJ, Eberhart CG, et al. Notch3 gene amplification in ovarian cancer. Cancer Res. 2006;66(12):6312–8. doi: 10.1158/0008- 5472.CAN-05-3610.
55. Perrone F, Bossi P, Cortelazzi B, Locati L, Quattrone P, Pierotti MA, Pilotti S, Licitra L. TP53 mutations and pathologic complete response to neoadjuvant cisplatin and fluorouracil chemotherapy in resected oral cavity squamous cell carcinoma. J Clin Oncol. 2010;28(5): 761–6. doi: 10.1200/JCO.2009.22.4170.
56. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, Olivier M. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat. 2007;28(6):622–9. doi: 10.1002/humu.20495.
57. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357(25):2552–61. doi: 10.1056/NEJMoa073770.
58. Poi MJ, Yen T, Li J, Song H, Lang JC, Schuller DE, Pearl DK, Casto BC, et al. Somatic INK4a-ARF locus mutations: a significant mechanism of gene inactivation in squamous cell carcinomas of the head and neck. Mol Carcinogen. 2001;30(1):26–36.
59. Qiu W, Schonleben F, Li X, Ho DJ, Close LG, Manolidis S, Bennett BP, Su GH. PIK3CA mutations in head and neck squamous cell carcinoma. Clin Cancer Res: Off J Am Assoc Cancer Res. 2006;12 (5):1441–6. doi: 10.1158/1078-0432.CCR-05-2173.
60. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nature Rev Cancer. 2003;3(10):756–67. doi: 10.1038/nrc1186.
61. Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H, Aster JC, Krishna S, et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J. 2001;20(13):3427–36. doi: 10.1093/emboj/20.13.3427.
62. Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, et al. High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res. 1996;56(16):3630–3.
63. Rocco JW, Ellisen LW. p63 and p73: life and death in squamous cell carcinoma. Cell Cycle. 2006;5(9):936–40.
64. Rocco JW, Sidransky D. p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res. 2001;264 (1):42–55. doi: 10.1006/excr.2000.5149.
65. Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res: Off J Am Assoc Cancer Res. 2006;12(17):5064–73. doi: 10.1158/1078-0432.CCR-06-0913.
66. Somers KD, Merrick MA, Lopez ME, Incognito LS, Schechter GL, Casey G. Frequent p53 mutations in head and neck cancer. Cancer Res. 1992;52(21):5997–6000.

67. Sopko R, McNeill H. The skinny on Fat: an enormous cadherin that regulates cell adhesion, tissue growth, and planar cell polarity. Curr Opin Cell Biol. 2009;21(5):717–23. doi: 10.1016/j.ceb.2009.07.001.
68. Soung YH, Lee JW, Kim SY, Jang J, Park YG, Park WS, Nam SW, Lee JY, et al. CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. Cancer Res. 2005;65(3): 815–21.
69. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333(6046):1157–60. doi: 10.1126/science.1208130.
70. Tanoue T, Takeichi M. New insights into Fat cadherins. J Cell Sci. 2005;118 (Pt 11):2347–53. doi: 10.1242/jcs.02398.
71. van Raam BJ, Salvesen GS. Proliferative versus apoptotic functions of caspase-8 Hetero or homo: the caspase-8 dimer controls cell fate. Biochimica et biophysica acta. 2012;1824(1):113–22. doi: 10.1016/j.bbapap.2011.06.005.
72. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, et al. The sequence of the human genome. Science. 2001;291(5507):1304–51. doi: 10.1126/science.1058040.
73. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, et al. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988;319(9):525–32. doi: 10.1056/NEJM198809013190901.
74. Wang MB, Billings KR, Venkatesan N, Hall FL, Srivatsan ES. Inhibition of cell proliferation in head and neck squamous cell carcinoma cell lines with antisense cyclin D1. Otolaryngol Head Neck Surg: Off J Am Acad Otolaryng Head Neck Surg. 1998;119(6):593–9.
75. Wang NJ, Sanborn Z, Arnett KL, Bayston LJ, Liao W, Proby CM, Leigh IM, Collisson EA, et al. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. Proc Natl Acad Sci U S A. 2011;108(43):17761–6. doi: 10.1073/pnas.1114669108.
76. Weinlich R, Dillon CP, Green DR. Ripped to death. Trends Cell Biol. 2011;21(11):630–7. doi: 10.1016/j.tcb.2011.09.002.
77. Welcker M, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nature Rev Cancer. 2008;8(2):83–93. doi: 10.1038/nrc2290.
78. Westfall MD, Pietenpol JA. p63: Molecular complexity in development and cancer. Carcinogenesis. 2004;25(6):857–64. doi: 10.1093/carcin/bgh148.
79. Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell. 1985;43(3 Pt 2):567–81.
80. White EA, Howley PM. Proteomic approaches to the study of papillomavirus-host interactions. Virology. 2013;435(1):57–69. doi: 10.1016/j.virol.2012.09.046.
81. Wreesmann VB, Singh B. Chromosomal aberrations in squamous cell carcinomas of the upper aerodigestive tract: biologic insights and clinical opportunities. J Oral Pathol Med: Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2005;34(8):449–59. doi: 10.1111/j.1600-0714.2005.00343.x.
82. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999;398(6729):714–8. doi: 10.1038/19539.
83. Yap TA, Workman P. Exploiting the cancer genome: strategies for the discovery and clinical development of targeted molecular therapeutics. Annu Rev Pharmacol Toxicol. 2012;52: 549–73. doi: 10.1146/annurev-pharmtox-010611-134532.
84. Yeudall WA, Crawford RY, Ensley JF, Robbins KC. MTS1/CDK4I is altered in cell lines derived from primary and metastatic oral squamous cell carcinoma. Carcinogenesis. 1994;15(12): 2683–6.
85. Young A, Lyons J, Miller AL, Phan VT, Alarcon IR, McCormick F. Ras signaling and therapies. Adv Cancer Res. 2009;102:1–17. doi: 10.1016/S0065-230X(09)02001-6.

86. Yu K, Toral-Barza L, Shi C, Zhang WG, Zask A. Response and determinants of cancer cell susceptibility to PI3K inhibitors: combined targeting of PI3K and Mek1 as an effective anticancer strategy. Cancer Biol Ther. 2008;7(2):307–15.
87. Zage PE, Nolo R, Fang W, Stewart J, Garcia-Manero G, Zweidler-McKay PA. Notch pathway activation induces neuroblastoma tumor cell growth arrest. Pediatr Blood Cancer. 2012;58(5):682–9. doi: 10.1002/pbc.23202.
88. Zhai C, Li Y, Mascarenhas C, Lin Q, Li K, Vyrides I, Grant CM, Panaretou B. The function of ORAOV1/LTO1, a gene that is overexpressed frequently in cancer: essential roles in the function and biogenesis of the ribosome. Oncogene. 2013. doi: 10.1038/onc.2012.604.
89. Zhang SY, Klein-Szanto AJ, Sauter ER, Shafarenko M, Mitsunaga S, Nobori T, Carson DA, Ridge JA, et al. Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck. Cancer Res. 1994;54(19):5050–3.
90. Zhang TH, Liu HC, Zhu LJ, Chu M, Liang YJ, Liang LZ, Liao GQ. Activation of Notch signaling in human tongue carcinoma. J Oral Pathol Med: Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2011;40(1):37–45. doi: 10.1111/j.1600-0714.2010.00931.x.
91. Zhao L, Vogt PK. Class I PI3K in oncogenic cellular transformation. Oncogene. 2008;27(41): 5486–96. doi: 10.1038/onc.2008.244.
92. Zweidler-McKay PA. Notch signaling in pediatric malignancies. Curr Oncol Rep. 2008;10(6): 459–68.
93. Zweidler-McKay PA, He Y, Xu L, Rodriguez CG, Karnell FG, Carpenter AC, Aster JC, Allman D, et al. Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. Blood. 2005;106(12):3898–906. doi: 10.1182/blood-2005-01-0355.

Chapter 16
Gene Expression in HNC

Michael F. Ochs and Joseph A. Califano

Abstract Head and Neck Cancer (HNC), which is most commonly Head and Neck Squamous Cell Carcinoma (HNSCC), shows substantial changes in gene transcription, as typical for other cancers. In contrast to many cancers, methylation, especially global hypomethylation and variable promoter methylation, plays a major role in control of expression in HNC. This methylation drives not only changes in expression levels but also changes in splicing. Here we discuss the present state of research in gene expression signatures, epigenetic regulation, microRNA expression, and alternative splicing in HNC.

Keywords Head and neck cancer · Human papilomavirus · Gene expression · MicroRNAs · Epigenetics · DNA methylation · Biomarkers

16.1 The Complexity of Gene Expression

Gene expression in mammalian organisms comprises the transcription of DNA into pre-mRNA, splicing and transport of pre-mRNA in the nucleus into mature mRNA in the cytoplasm and endoplasmic reticulum, and translation of the mRNA into protein by ribosomes. This process is further modified by the interaction of mRNA with microRNA (miR), processed by the DICER complex, which leads to destruction of mRNA in mammals and subsequent loss of protein production. The transcription of DNA is heavily modified by epigenetic changes, especially promoter methylation, which is critically important to the etiology of head and neck cancers (HNC).

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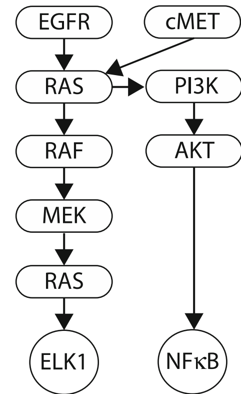

Fig. 16.1 A highly simplified view of important signaling pathways in HNC, highlighting the fact that expression, governed by transcription factors shown as *circles,* is best viewed as a downstream effect of receptor activation and signaling activity

This review will focus on several aspects of gene expression in HNC. First, we will review the transcriptomic signatures, especially those related to biological processes and prognosis, that have been reported. Second, we will review the growing understanding of the role of epigenetics in activation of oncogenes and silencing of tumor suppressors. Third, we will discuss the inference on transcriptional regulators and the association of these regulators with human papillomavirus (HPV) status and with signaling processes. Fourth, we will discuss two emerging areas—the roles of miRs and of alternative splice variants.

16.2 Expression Profiling

It is useful to begin with a brief consideration of the biology of gene regulation in cancer to focus the discussion on key points where we can leverage transcriptomic data to gain insight. Gene expression is often the downstream consequence of changes in cellular states and signaling pathway activities. As such, it can provide a point of data unification for a cancer cell, since aberrations in many different signaling proteins can lead to activation of the same transcriptional response (see Fig. 16.1, and Chapters 6–8). As it is clear from studies in a number of cancers that pathways tend to be disrupted universally, but mutations in different proteins drive the disruption in different individuals [1], [2], transcriptomics cannot generally identify the driver mutation of this activity. While there are exceptions to this general case, especially for receptor tyrosine kinases (RTKs, see Chapters 3–5), we will focus here on transcriptional signatures that relate to prognosis and which may be considered as integrating many potential upstream changes across individual patients.

The emergence of microarray technology in the 1990s [3], [4] coupled to the development of robust statistical methods for normalization [5] and classification [6] has led to improved insight into changes in gene expression in HNC. Efforts have focused on both identification of prognostic signatures, similar to the Oncotype DX and MammaPrint efforts in breast cancer [7], and elucidation of biological processes.

In one of the seminal studies of gene expression signatures in HNC, Chung et al. applied hierarchical clustering to a set of 60 tumor samples measured using Agilent cDNA arrays and identified four subtypes [8]. One group was associated with high transforming growth factor alpha (TGFα) expression and showed significantly poorer survival. As with other early studies using hierarchical clustering, a direct transition to a biomarker would not have been easy; however, this study did establish that molecular subtypes of HNC exist and reflect outcomes.

With the increasing incidence of HPV+ HNC, there is a need to elucidate the molecular similarities and differences of HPV+ and HPV− tumors. Schlecht et al. identified 123 genes whose expression was significantly different between HPV+ and HPV− tumors, including upregulation of genes associated with cervical cancer in HPV+ cases: cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4; CDKN2C), replication factor C (activator 1) 4, 37 kDa (RFC4), and transcription factor DP2 (E2F dimerization partner; TFDP2) [9].

The importance of stage in outcome for HNC led to significant effort to identify a diagnostic expression signature of nodal metastasis. Roepman et al. used an 82 tumor set to isolate a 102 gene signature, which correctly determined node involvement with an accuracy of 74 % [10]. Using the same data, Hensen et al. identified 26 genes significantly differentially expressed between HNC with and without nodal metastases. They validated these genes in a 22 tumor set using a different microarray platform; however, only seven of the 21 genes available on the second platform validated, and none survived multiple testing corrections [11]. The lack of validation may reflect the small number of tumors, or tumor or hypoxic state, since the seven genes include metalloproteases, and some had functions linked to hypoxia, and angiogenesis (see Chapter 14, Kuo and Le).

Roepman et al. followed their initial study with a study of 19 tumor and lymph node metastasis pairs targeted at identifying genes with changed expression [12]. Applying statistical analysis of microarrays (SAM) [13] identified only a single gene at a false discovery rate (FDR) of 5 %, metastasis-associated gene 1 (MTA1). In a study of 40 paired primary HNC tumors and cervical metastases, Liu et al. identified 301 differentially expressed genes and validated by immunohistochemistry upregulation of selectin L (SELL) and downregulation of insulin-like growth factor binding protein 6 (IGFBP6) in metastases [14]. The differences in the number of genes identified in these similar studies may be due to the weaker statistical test applied by Liu et al. and the smaller number of samples in Roepman et al., which also showed low tumor cellularity in most cases. The impact of different statistical analysis methods is highlighted in work by Braackhuis et al., where they demonstrated that 150 genes identified in a *t*-test were not identified by SAM when searching for metastases [15].

Another area of great interest for biomarkers is determination of initial treatment response and prediction of survival, and several studies looked at recurrence as an endpoint. Chung et al. generated a signature using formalin-fixed paraffin-embedded (FFPE) and frozen tumor pairs and validated it on previously published frozen tissue data [16]. They identified a 75 gene signature associated with recurrence which was enriched for genes involved in epithelial-mesenchymal transformation (EMT) and in nuclear factor kappa B (NFκB) signaling. Previously, Ginos et al. had shown that there were differences between recurrent tumors and primary tumors, specifically in

the expression of genes associated with invasion [17], which is qualitatively similar to EMT. Thurlow et al. used spectral clustering to look at sets defined by gene ontology [18] and demonstrated that the functional assignment of focal adhesion-related provided a gene set prognostic of poor outcome [19]. Pavón et al. applied clustering to a set of 63 pretreatment biopsies and identified a cluster associated with poorer local recurrence-free survival, progression-free survival, and overall survival [20]. This cluster was also associated with cell adhesion and NFκB and integrin signaling, in accord with the other studies.

Pramana et al. analyzed 92 pretreatment biopsies for genes predictive of response to chemoradiation using samples split into training and test sets [21]. While no genes validated in terms of response across the two sets, they did validate the signature from Chung et al. noted above [16]. Dumur et al. analyzed a set of microarrays generated from pretreatment biopsies of eight complete responders and six nonresponders to radiation therapy, identifying a set of 167 probe sets that were significantly different between the two sets [22].

Although there has been considerable effort expended on identifying expression biomarkers for prognosis and treatment response, the identification of a clinically useful marker has not yet occurred. This parallels the experience in other cancers, where the cost and difficulty of the use of expression-based markers has hindered development of an appropriate test [23]–[25]. A detailed recent review of molecular approaches in HNC provides additional detail [26], including the use of proteomics in biomarker development [27], [28].

As noted in Fig. 16.1, one important issue that has not been addressed in most analyses is that expression is generally a downstream indicator of signaling activity, which drives processes (e.g., NFκB and integrin signaling, EMT) that have been identified as important in HNC. The endpoints of signaling pathways are typically transcription factors (TFs), suggesting that use of knowledge of TF targets may aid analysis of signaling activity by treating changes in levels of TF targets as a surrogate for TF activity [29]. Recent work by Fertig et al. in cell line studies suggests that this may be a useful approach for HNC [30].

16.3 Epigenetic Regulation of Oncogenes and Tumor Suppressors

Perhaps the greatest indicator that epigenetic alteration is a significant factor in expression alterations paradoxically comes from genetic studies. The results of whole genome and exome sequencing in head and neck squamous cell carcinoma (HNSCC) have been recently reported, and surprisingly, a large proportion of mutation targets found had already been previously described (TP53, CDKN2A, PIK3CA, PTEN, and HRAS) and only 30 % of tumors contained a mutation in a novel target gene [31], [32]. Additional discovery of genetic alterations, including mutation in noncoding regions as well as chromosomal loss and gain, may yield additional gene targets involved in carcinogenesis; however, sequencing data do not account for the extensive nature of gene expression alterations in HNSCC. Increasingly, epigenetic alteration is found to affect gene expression for multiple tumor suppressor gene and oncogene targets.

The dominant paradigm of abnormal epigenetic regulation in cancers includes CpG island promoter hypermethylation that results in tumor suppressor gene transcriptional repression and has been described for an extensive number of gene targets [33]. In addition, investigators have defined hypomethylated proto-oncogenes in HNSCC. These strategies have defined gene specific targets for opportunities for therapeutic intervention, as well as useful biomarkers for detection and prognosis [34]–[36]. With the advent of a variety of high-throughput discovery techniques, there has been an explosion in the identification of gene promoters demonstrating differential hypermethylation in HNSCC, involving essentially all the important regulatory pathways noted to be altered in HNSCC, which have been discussed in detail recently [37], [38]. While fruitful, these approaches fail to account for most expression alterations and have essentially ignored the larger proportion of cancer-associated methylation alterations in HNSCC outside of promoter regions. In addition, global genomic hypomethylation has been almost universally noted in solid tumors, including oral squamous cell carcinoma (OSCC) [39]. For the past decade, the dominant paradigm for study of epigenetic alteration in human malignancy has focused on repression of tumor suppressor genes by epigenetic changes including promoter methylation and histone acetylation [40].

These data suggest that promoter-based epigenetic and DNA methylation alterations in most cancers, including HNSCC, may be only a part of the genomic landscape of methylation alterations. Implicit in these data are indications that genomic methylation alterations outside of promoter region CpG islands likely play a significant role in carcinogenesis, and may include gene-specific effects as well as genome-wide effects.

Recently, approaches have been reported in other solid tumor systems that allow whole-genome methylation analysis that includes intragenic and intergenic regions outside of promoter regions. These studies provide novel insights into epigenetic regulation of gene expression due to the comprehensive nature of the genomic analysis of DNA methylation. In addition, these approaches challenge the dominant paradigm that CpG island promoter methylation is the sole determinant of aberrant, locus-specific gene expression. Furthermore, the landscape of cancer-specific methylation alterations has expanded in scope, such that additional areas beyond CpG promoter islands, including demethylated blocks, CpG shores, intragenic regions, intergenic regions, and repetitive elements, are described as demonstrating differentially methylated regions (DMRs) in human cancers and contributing to transcriptional deregulation.

The approaches that resulted in selective emphasis of promoter hypermethylation abnormalities have often relied on techniques that provide limited approximations of methylation status, either by relying on interrogation of a simplified representation of the methylation status that includes a small fraction of CpGs in the genome, reliance on indirect measurement of expression after pharmacologic demethylation in model systems, or by exclusive examination of promoter regions.

Paradoxically, global genomic hypomethylation has been reported in almost all solid tumors, and this phenomenon occurs in HNSCC [41]–[43]. In solid human tumors, meta-analysis of DNA global hypomethylation primary tumor data shows

an overall correlation between global hypomethylation and advanced tumor stage, and we have confirmed this observation in HNSCC [39]. A variety of hypotheses have been developed regarding the functional effects of genomic hypomethylation, including induction of aneuploidy due to demethylation of centromeric regions, epigenetic activation of proto-oncogenes, and disruption of DNA structure resulting in chromosomal instability via reactivation of transposable elements. Using pharmacologically demethylated cell lines combined with cancer outlier profile analysis (COPA) in primary tissues, sets of candidate proto-oncogenes that undergo aberrant demethylation and increased expression in primary human tumors were identified in HNSCC and non-small cell lung carcinoma (NSCLC) [44], [45]. These data indicated that aberrant demethylation of multiple, physiologically repressed proto-oncogenes and cancer testis antigens occurs in human cancers in a coordinated fashion in individual tumors and implicates the specific transcription factors, BORIS and CTCF, in this regulation. This strategy is limited by cell line artifacts, as well as use of an indirect methodology to survey methylated promoters using pharmacologic unmasking. In addition, a large amount of DNA outside of gene-specific promoter regions is likely also affected by derangements in methylation status. These regions may include CpG island shores, intragenic, and 3′ regions associated with specific genes, as well as large intergenic regions and repetitive elements.

Recent data using whole genome bisulfite sequencing in primary colon cancers have also shown that large genomic stretches of relative hypomethylation are associated with variable increased gene expression for large sets of adjacent genes, indicating a potential alternative method of gene deregulation that may be far more common than CpG promoter island hypermethylation [46]. Together these studies demonstrate that perhaps even the majority of CpG methylation-related expression alterations in solid cancers may be unrelated to CpG promoter hypermethylation.

Whole Genome Methylation Analysis in Other Solid Tumors Original reports on the use of methylated DNA immunoprecipitation (MeDIP) employed analysis of tumor-derived cell lines and were primarily focused on discovery of aberrant promoter methylation patterns with or without validation in primary tumors [47]–[50]. Subsequent reports and technical advances, including higher density arrays and genome-wide amplification, facilitated more complete genomic array analyses in normal tissues [51] and primary lung cancers [52]. These two cited studies employed MIRA, which includes selective enrichment using methylated DNA-binding proteins and genome amplification followed by array hybridization. These studies demonstrated several interesting findings, including a significant level of subtelomeric hypomethylation and short interspersed elements (SINE) and long interspersed elements (LINE) promoter hypomethylation associated with lung cancers, and demonstrated a broad and accurate ability to define multiple lung cancer-specific hypermethylated gene promoter regions. This group also examined methylation in normal human B cells using a high density array, and noted that methylation of promoters was associated with low-gene expression, but exceptions to this finding were due to transcription from cryptic start sites and expression was related to alternate splice variant expression from these start sites. In addition, methylation of

intragenic sequences was correlated with increased gene expression, and frequent methylation of 3′ end of genes was noted. These data suggest broader physiologic or pathologic roles for CpG methylation marks, including induction of alternate splice variants due to cryptic start sites located within intragenic regions (see Sect. 16.5, on alternative splicing), inhibition of intragenic transcriptional activity, and induction of genetic instability through inappropriate methylation marks in telomere regions, transposable elements, or at regions predisposed to genomic amplification.

A study examining primary colon cancers using comprehensive high-throughput arrays for relative methylation (CHARM) to examine genomic DNA methylation demonstrated that the methylation status of low-density CpG areas located within 2 kb of promoter-associated CpG islands, termed CpG shores, had a higher correlation with gene expression than the CpG islands themselves [53]. A follow-up study employing whole genome bisulfite sequencing in primary colon cancers showed up to 25 % hypomethylation of discrete 5–10 kb blocks encompassing half the genome, with extreme gene expression variability for the genes contained within these blocks [46]. Ironically, only a small fraction of differential methylation was related to areas of hypermethylation, which has been the dominant focus of epigenetic investigation in solid tumors. Other smaller DMRs showed a loss of sharply delimited methylation boundaries at CpG islands. This study did not directly measure expression differences within tumors that underwent whole genome bisulfite sequencing, but used published array-based expression data to show that areas encompassed by hypomethylated blocks undergo highly variable increased expression, in general, in colon cancers.

These data also raise the issue as to what level of partial methylation alteration is associated with transcriptional deregulation. For example, large hypomethylated blocks have variable increased expression and only undergo partial demethylation [46], but direct simultaneous measurement of expression in these regions in primary tumors has not been reported. Performance of these studies would be expected to provide more precise definition of the transcriptional effects associated with variable levels of differential methylation and the effect of a loss of methylation boundaries on genes at the interface of these boundaries.

Available Techniques and Platforms for Genome-Wide Analyses of DNA Methylation Shotgun whole genome sequencing of bisulfite-treated DNA has been performed in a mammalian genome and represents a gold standard for determination of methylation status at a single nucleotide level [54]. With constrained resources, most investigators employ enrichment or modification of methylated DNA, followed by high-throughput array hybridization or high-throughput genomic sequencing. Commercial applications often cover a small fraction of methylated bases using array hybridization techniques, so investigators have developed alternative applications. One report has attempted to directly compare methods, and found “bias toward CpG islands in MeDIP, relatively incomplete coverage in HELP (HpaII tiny fragment enrichment by ligation-mediated PCR), and location imprecision in McrBC analysis” [55]. Recent sequencing strategies, including reduced representation bisulfite sequencing (RRBS), facilitate coverage of a large portion of CpG sites but have a likely

bias toward improved coverage of CpG dense sites [56]. Of note, characterization of the largest cancer-associated contribution toward genomic hypomethylation in colon cancer, variably hypomethylated blocks up to 10 kb in size, was not discovered until whole genome bisulfite sequencing was employed [46]. However, there are novel techniques that are reported regularly, and it is expected that as coverage and fidelity increases, costs will continue to decrease.

HPV-Positive HNC and Methylation Early epigenetic investigation of RASSF1A promoter methylation indicated a significant inverse correlation between RASSF1A promoter methylation and HPV infection [57]. It is widely observed that whole genome hypomethylation is a general characteristic of solid tumors and is accompanied by gene specific promoter region hypermethylation. The observation that epigenetic alteration is decreased in HPV-positive HNSCC was confirmed by other investigators who found that LINE hypomethylation was more pronounced in HPV-negative than in HPV-positive tumors. Genomic instability, as measured by genome-wide loss-of-heterozygosity single nucleotide polymorphism (SNP) analysis, was greater in HNSCC samples with more pronounced LINE hypomethylation [58]. As a whole, these data indicate that HPV-positive HNSCC has fewer mutational, genetic, and chromosomal alterations, and a different composition of epigenetic alterations compared with non-HPV-related HNSCC. The Cancer Genome Atlas (TCGA) has been conducting high-throughput analyses of HNSCC. Drs. Liudmila Danilova and Leslie Cope (personal communication) have analyzed preliminary data on 292 tumors using the Infinium Human Methylation 450K platform and indicate that analysis of 30 HPV-positive HNSCC and 207 HPV-negative HNSCC show significant epigenetic differences, and that HPV-positive HNSCC has higher average methylation levels over frequently methylated loci compared to HPV-negative HNSCC [59].

In summary, the understanding of the nature of epigenetic alteration in HNSCC is at an early stage. Advances in sequencing are expected to increase the number of genes identified as targeted by methylation alterations, but also to extend our understanding of the epigenetic contribution from genomic areas outside of promoter regions to HNSCC development. Further areas that are relatively unexplored include the contribution of other epigenetic changes, including histone alterations and alterations in epigenetic regulatory components to HNSCC development.

16.4 MicroRNA Expression

Since the identification of small noncoding RNAs that disrupted gene expression in *Caenorhabditis elegans* development [60], the study of miRs has led to identification of numerous roles in development and disease [61]–[63]. In cancer, miRs appear to play important roles as both oncogenes and tumor suppressors, and notably miRs playing both roles have been identified in HNCs [64]. The transformative properties of miRs, both in promotion of initial tumor formation and in EMT, suggest that identification of deregulated miRs and their targets will help elucidate properties essential to tumor growth and aid in identification of targets for treatment.

Table 16.1 MicroRNAs that were detected as differentially expressed in at least three of eight genome-wide studies of head and neck cancer

Regulation	Concordant miRs (minimum of 3 of 8 studies)
Upregulated in HNC	miR-21, miR-155, miR-31, miR-223
Downregulated in HNC	miR-375, miR-1, miR-133a, miR-99a, miR-125b, miR-100, miR-143, miR-204

In HNCs, the role of miRs has been studied extensively over the last decade. A number of miRs functioning as tumor suppressors and oncogenes have been identified; however, agreement between studies is weak. In a recent review of the field [65], concordance was defined as only three of eight genome-wide screening studies reporting a miR as deregulated; however, only 12 miRs were then considered deregulated (see Table 16.1). The inconsistent results are likely the result of application of different technologies and small sample sizes. A similar lack of concordance was seen in the early days of expression microarrays, before proper normalization and artifact detection methodologies were developed.

The studies of miRs in HNC have included both cell line studies that have looked at potential functional analyses and tissue studies that have aimed primarily at biomarker discovery. Here we will focus on the tissue studies.

In a small study, Chang et al. used 4 HNC and 4 uvulopalatopharyngoplasty (UPPP) samples to identify candidate deregulated miRs using SAM [66]. Candidate miRs were then validated in 27 HNC and 8 UPPP by RT-PCR and confirmed to be functional in cell line studies, yielding miR-21 and miR-494 as candidates.

Childs et al. aimed to develop a miR-based prognostic marker of survival in a study comprising 104 patients [67]. They identified low expression of let-7d and miR-205 as prognostic markers with a hazard ratio of 4.6 independent of clinical covariates. However, these markers were not validated. In addition, it is not clear whether there is a clinical benefit to such a marker, since the functional consequence of loss of expression is not known and therefore there is no clear clinical intervention.

In a similar study, Avissar et al. used array profiling to measure expression of 662 miRs in 16 HNC and 5 head and neck epithelial controls [68]. SAM analysis identified 12 human miRs with significantly altered expression at an FDR of 0.0001. Four miRs were validated by RT-PCR at $p < 0.01$, including miR-21, miR-18a, and miR-221 as upregulated in HNC and miR-375 as downregulated. Forming ratios and adding 83 HNC and 9 normal additional samples yielded miR-221/miR-375 as a biomarker with sensitivity of 0.92 and specificity of 0.93 for identification of HNC. Unfortunately, the experimental design mixed the discovery and confirmation sets, so the final measurements cannot be considered to provide independent confirmation. In addition, the measurements were done on tumor samples, and a biomarker would only be useful if it identified cancer prior to discovery by other means (e.g., from oral brush samples or in serum).

Hui et al. performed a similar genome-wide screen of miRs [69]. Interestingly, they validated functional effects of miR-375 loss and the miR-106b-25 cluster gain by overexpressing miR-375 and knocking down the cluster, which led to loss of proliferation in cell lines. With follow-up data on relapse, they also identified miR-451 as a potential biomarker of relapse.

Gee et al. looked into the use of miRs as biomarkers of hypoxia, given the predicted regulation of miR-210, miR-21, and miR-10b in hypoxic conditions [70]. In HNSCC cell lines grown under hypoxic conditions, only miR-210 showed significant induction. Analysis was then performed on 46 HNC samples and ten normal adjacent tissue samples. Expression of miR-21 and miR-10b was significantly different between tumor and normal tissue, while miR-210 showed no significant difference. However, miR-210 alone showed correlation with the hypoxic metagene previously defined [71]. Given the stability of miRs, they propose that miR-210 may therefore be a more convenient biomarker of hypoxia.

An additional potential link to hypoxia was proposed by Liu et al. for miR-31 [72]. Using ten HNSCC with matched normal tissues, they identified miR-31 as the strongest deregulated miR out of 154 measured by qRT-PCR and demonstrated that blocking its expression in HNSCC cell lines reduced migration. They identified factor inhibiting HIF-1α (FIH) as a target of miR-31 and showed its loss of expression in tumors.

More recently, the investigations have shifted to functional and validation studies of miRs identified in early genome-wide screens. Lo et al. focused on aldehyde dehydrogenase 1 (ALDH1) + /CD44 + cells isolated from HNC tumors [73]. They found that miR-200c was downregulated and that site-directed mutagenesis showed direct interaction of miR-200c with BMI1 polycomb ring finger oncogene, which was upregulated in the ALDH1 + /CD44 + cells.

Nohata et al. followed up the discovery of the strong downregulation of miR-375 in HNC with functional studies showing that restoration of miR-375 expression induced apoptosis in HNC cell lines [74]. Combining expression microarray measurements in SAS and FaDu cell lines with TargetScan 3′ UTR prediction identified 16 potential miR-375 targets. Limiting further study to genes more strongly affected than a known target, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), they identified metadherin (MTDH) as a target and demonstrated that silencing of this gene reduced growth of SAS and FaDu cells.

Meta-analyses of previous studies confirm changes in the miR-99 family [75] and differential miR changes in HPV-positive and HPV-negative HNSCC [76].

Overall miR measurements show great promise as potential biomarkers in HNC, including as predictors of the probability of recurrence, and for determination of hypoxia and early detection. One major advantage to miR biomarkers over expression biomarkers is the stability of miRs, which make them available for measurement in bodily fluids, including saliva and serum. For instance, potential serum miR biomarkers have been identified in lung cancer [77].

As with gene expression, it is expected that miR expression will be partially regulated by methylation changes. Early evidence for this in HNC has been presented for miR-9 [78], and the differential expression seen in other miRs is likely to include methylation drivers given the widespread methylation changes seen in HNC.

16.5 Alternative Splice Variants

Alternative splicing plays an important role in gene expression, permitting multiple protein isoforms to be encoded within a single DNA gene. Splice variants have been identified that play a causative role in cancer [79]. As methylation of the intragenic and promoter areas has been known to drive alternative splicing [80], there is reason to believe that the potential impact of splice variants in HNC may be high given the significant methylation changes associated with smoking (see Sect. 16.3 on epigenetic regulation).

Among the earliest splicing isoforms linked to cancer were variants of the CD44 gene, which is a surface glycoprotein associated with cell adhesion. CD44 variants were identified in HNC in the early 1990s [81]; however, subsequent studies showed that many isoforms exist in different normal head and neck tissues with a diverse distribution [82], suggesting that changes associated with carcinomas may arise from overall expression changes rather than alternative splicing.

A common splice variant of cyclin D1 (CCND1), G870A, has been associated with higher risk of developing HNC. In a study of 698 HNC cases and 777 controls, G870A germline homozygotes had higher risk of HNC (odds ratio 1.5) but better overall survival [83]. The effect is believed due to the greater stability of protein leading to increased G1-S transition rates. Interestingly, the G870A splice variant has also been shown to have a synergistic effect with splice variants of the TFIIH complex member excision repair cross-complementing rodent repair deficiency, complementation group 2 (XPD/ERCC2) in a case-control study of 273 cases and 269 controls (odds ratio of 7.09) [84].

Moller-Levet et al. identified a splice variant of laminin α 3 (LAMA3) as associated with HNC [85]. The variant, LAMA3-A, appeared to be associated with hypoxia, while the full-length isoform, LAMA3-B, was not. In a novel cohort, high expression of LAMA3-A was associated with poorer survival following surgery with standard adjuvant treatment.

Additional alternative splicing events have been identified in cell lines studies, though they have yet to be confirmed in tumors. Cai et al. identified three separate vascular endothelial growth factor A (VEGF) variants, although HNC seemed associated with overexpression instead of specific isoforms [86]. Mao et al. reported on exon deletions in fragile histidine triad (FHIT), a tumor suppressor gene, in cell lines [87]. As FHIT lies in cytoband 3p14, which often shows loss of heterozygosity, it is possible that these deletions represent a second hit on FHIT.

References

1. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu

VE, Kinzler KW. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008;321(5897):1807–12.

2. TCGA_Consortium. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008;455(7216):1061–8.
3. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol. 1996;14(13):1675–80.
4. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270(5235):467–70.
5. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics (Oxford England). 2003;4(2):249–64.
6. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3(Article 3)
7. Ross JS. Multigene classifiers, prognostic factors, and predictors of breast cancer clinical outcome. Adv Anat Pathol. 2009;16(4):204–15.
8. Chung CH, Parker JS, Karaca G, Wu J, Funkhouser WK, Moore D, Butterfoss D, Xiang D, Zanation A, Yin X, Shockley WW, Weissler MC, Dressler LG, Shores CG, Yarbrough WG, Perou CM. Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. Cancer Cell. 2004;5(5):489–500.
9. Schlecht NF, Burk RD, Adrien L, Dunne A, Kawachi N, Sarta C, Chen Q, Brandwein-Gensler M, Prystowsky MB, Childs G, Smith RV, Belbin TJ. Gene expression profiles in HPV-infected head and neck cancer. J Pathol. 2007;213(3):283–93. doi:10.1002/path.2227.
10. Roepman P, Wessels LF, Kettelarij N, Kemmeren P, Miles AJ, Lijnzaad P, Tilanus MG, Koole R, Hordijk GJ, van der Vliet PC, Reinders MJ, Slootweg PJ, Holstege FC. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. Nat Genet. 2005;37(2):182–6. doi:10.1038/ng1502
11. Hensen EF, De Herdt MJ, Goeman JJ, Oosting J, Smit VT, Cornelisse CJ, Baatenburg deJRJ. Gene-expression of metastasized versus non-metastasized primary head and neck squamous cell carcinomas: a pathway-based analysis. BMC Cancer. 2008;8:168. doi:10.1186/1471-2407-8-168.
12. Roepman P, de Jager A, Groot Koerkamp MJ, Kummer JA, Slootweg PJ, Holstege FC. Maintenance of head and neck tumor gene expression profiles upon lymph node metastasis. Cancer Res. 2006;66(23):11110–4. doi:10.1158/0008-5472.CAN-06-3161
13. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 2001;98(9):5116–21.
14. Liu CJ, Liu TY, Kuo LT, Cheng HW, Chu TH, Chang KW, Lin SC. Differential gene expression signature between primary and metastatic head and neck squamous cell carcinoma. J Pathol. 2008;214(4):489–97. doi:10.1002/path.2306.
15. Braakhuis BJ, Senft A, de Bree R, de Vries J, Ylstra B, Cloos J, Kuik DJ, Leemans CR, Brakenhoff RH. Expression profiling and prediction of distant metastases in head and neck squamous cell carcinoma. J Clin Pathol. 2006;59(12):1254–60. doi:10.1136/jcp.2005.035451
16. Chung CH, Parker JS, Ely K, Carter J, Yi Y, Murphy BA, Ang KK, El-Naggar AK, Zanation AM, Cmelak AJ, Levy S, Slebos RJ, Yarbrough WG. Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-kappaB signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer Res. 2006;66(16):8210–8. doi:10.1158/0008-5472.CAN-06-1213.
17. Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE, Ondrey FG, Adams GL, Gaffney PM. Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res. 2004;64(1):55–63.
18. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25–9.

19. Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G, West CM, Harris AL, Parkinson EK, Harrison PR, Ozanne BW, Partridge M, Kalna G. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. J Clin Oncol. 2010;28(17):2881–8. doi:10.1200/JCO.2009.24.8724.
20. Pavon MA, Parreno M, Tellez-Gabriel M, Sancho FJ, Lopez M, Cespedes MV, Casanova I, Lopez-Pousa A, Mangues MA, Quer M, Barnadas A, Leon X, Mangues R. Gene expression signatures and molecular markers associated with clinical outcome in locally advanced head and neck carcinoma. Carcinogenesis. 2012;33(9):1707–16. doi:10.1093/carcin/bgs207.
21. Pramana J, Van den Brekel MW, van Velthuysen ML, Wessels LF, Nuyten DS, Hofland I, Atsma D, Pimentel N, Hoebers FJ, Rasch CR, Begg AC. Gene expression profiling to predict outcome after chemoradiation in head and neck cancer. Int J Radiat Oncol Biol Phys. 2007;69(5):1544–52. doi:10.1016/j.ijrobp.2007.08.032.
22. Dumur CI, Ladd AC, Wright HV, Penberthy LT, Wilkinson DS, Powers CN, Garrett CT, DiNardo LJ. Genes involved in radiation therapy response in head and neck cancers. Laryngoscope. 2009;119(1):91–101. doi:10.1002/lary.20005.
23. Gold KA, Kim ES. Role of molecular markers and gene profiling in head and neck cancers. Curr Opin Oncol. 2009;21(3):206–11. doi:10.1097/CCO.0b013e328329ac00.
24. Makitie AA, Monni O. Molecular profiling of laryngeal cancer. Expert Rev Anticancer Ther. 2009;9(9):1251–60. doi:10.1586/era.09.102.
25. Braakhuis BJ, Brakenhoff RH, Leemans CR. Gene expression profiling in head and neck squamous cell carcinoma. Curr Opin Otolaryngol Head Neck Surg. 2010;18(2):67–71. doi:10.1097/MOO.0b013e32833693ce.
26. Sahu N, Grandis JR. New advances in molecular approaches to head and neck squamous cell carcinoma. Anti-cancer Drugs. 2011;22(7):656–64. doi:10.1097/CAD.0b013e32834249ba.
27. Byers LA, Holsinger FC, Kies MS, William WN, El-Naggar AK, Lee JJ, Hu J, Lopez A, Tran HT, Yan S, Du Z, Ang KK, Glisson BS, Raso MG, Wistuba II, Myers JN, Hong WK, Papadimitrakopoulou V, Lippman SM, Heymach JV. Serum signature of hypoxia-regulated factors is associated with progression after induction therapy in head and neck squamous cell cancer. Mol Cancer Ther. 2010;9(6):1755–63. doi:10.1158/1535-7163.MCT-09-1047.
28. Ralhan R. How will increasing our knowledge of the head and neck cancer cells' secretome benefit head and neck squamous cell carcinoma patients? Expert Rev Proteomics. 2012;9(2):115–8. doi:10.1586/epr.12.1.
29. Ochs MF, Rink L, Tarn C, Mburu S, Taguchi T, Eisenberg B, Godwin AK. Detection of treatment-induced changes in signaling pathways in gastrointestinal stromal tumors using transcriptomic data. Cancer Res. 2009;69(23):9125–32. doi:10.1158/0008-5472.CAN-09-1709.
30. Fertig EJ, Ren Q, Cheng H, Hatakeyama H, Dicker AP, Rodeck U, Considine M, Ochs MF, Chung CH. Gene expression signatures modulated by epidermal growth factor receptor activation and their relationship to cetuximab resistance in head and neck squamous cell carcinoma. BMC Genomics. 2012;13:160. doi:10.1186/1471-2164-13-160.
31. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs RA, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333(6046):1154–7.
32. Stransky N, Vallot C, Reyal F, Bernard-Pierrot I, de Medina SG, Segraves R, de Rycke Y, Elvin P, Cassidy A, Spraggon C, Graham A, Southgate J, Asselain B, Allory Y, Abbou CC, Albertson DG, Thiery JP, Chopin DK, Pinkel D, Radvanyi F. Regional copy number-independent deregulation of transcription in cancer. Nat Genet. 2006;38(12):1386–96.
33. Ha PK, Califano JA. Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. Lancet Oncol. 2006;7(1):77–82.
34. Pattani KM, Zhang Z, Demokan S, Glazer C, Loyo M, Goodman S, Sidransky D, Bermudez F, Jean-Charles G, McCaffrey T, Padhya T, Phelan J, Spivakovsky S, Bowne HY, Goldberg

JD, Rolnitzky L, Robbins M, Kerr AR, Sirois D, Califano JA. Endothelin receptor type B gene promoter hypermethylation in salivary rinses is independently associated with risk of oral cavity cancer and premalignancy. Cancer Prev Res (Phila). 2010;3(9):1093–103. doi:10.1158/1940-6207.CAPR-10-0115.
35. Carvalho AL, Jeronimo C, Kim MM, Henrique R, Zhang Z, Hoque MO, Chang S, Brait M, Nayak CS, Jiang WW, Claybourne Q, Tokumaru Y, Lee J, Goldenberg D, Garrett-Mayer E, Goodman S, Moon CS, Koch W, Westra WH, Sidransky D, Califano JA. Evaluation of promoter hypermethylation detection in body fluids as a screening/diagnosis tool for head and neck squamous cell carcinoma. Clin Cancer Res. 2008;14(1):97–107.
36. Carvalho AL, Henrique R, Jeronimo C, Nayak CS, Reddy AN, Hoque MO, Chang S, Brait M, Jiang WW, Kim MM, Claybourne Q, Goldenberg D, Khan Z, Khan T, Westra WH, Sidransky D, Koch W, Califano JA. Detection of promoter hypermethylation in salivary rinses as a biomarker for head and neck squamous cell carcinoma surveillance. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2011;17(14):4782–9. doi:10.1158/1078-0432.CCR-11-0324.
37. Demokan S, Dalay N. Role of DNA methylation in head and neck cancer. Clin Epigenetics. 2011;2(2):123–50. doi:10.1007/s13148-011-0045-3.
38. Stephen JK, Chen KM, Havard S, Harris G, Worsham MJ. Promoter methylation in head and neck tumorigenesis. Methods Mol Biol. 2012;863:187–206. doi:10.1007/978-1-61779-612-8_11.
39. Smith IM, Mydlarz WK, Mithani SK, Califano JA. DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. Int J Cancer. 2007;121(8):1724–8.
40. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128(4):683–92.
41. Das PM, Singal R. DNA methylation and cancer. J Clin Oncol. 2004;22(22):4632–42.
42. Dunn BK. Hypomethylation: one side of a larger picture. Ann N Y Acad Sci. 2003;983:28–42.
43. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene. 2002;21(35):5400–13.
44. Glazer CA, Smith IM, Ochs MF, Begum S, Westra W, Chang SS, Sun W, Bhan S, Khan Z, Ahrendt S, Califano JA. Integrative discovery of epigenetically derepressed cancer testis antigens in NSCLC. PloS One. 2009;4(12):e8189. doi:10.1371/journal.pone.0008189.
45. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, Vostrov A, Abdullaev Z, Lobanenkov V, Gray A, Liu C, Chang SS, Ostrow KL, Westra WH, Begum S, Dhara M, Califano J. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PloS One. 2009;4 (3):e4961. doi:10.1371/journal.pone.0004961.
46. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP. Increased methylation variation in epigenetic domains across cancer types. Nat Genet. 2011;43(8):768–75. doi:10.1038/ng.865.
47. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet. 2005;37(8):853–62. doi:10.1038/ng1598.
48. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I. Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet. 2006;38 (2):149–53. doi:10.1038/ng1719.
49. Cheung HH, Lee TL, Davis AJ, Taft DH, Rennert OM, Chan WY. Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. Br J Cancer. 2010;102(2):419–27. doi:10.1038/sj.bjc.6605505.
50. Weng YI, Huang TH, Yan PS. Methylated DNA immunoprecipitation and microarray-based analysis: detection of DNA methylation in breast cancer cell lines. Methods Mol Biol. 2009;590:165–76. doi:10.1007/978-1-60327-378-7_10.
51. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP. A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci U S A. 2009;106(3):671–78. doi:10.1073/pnas.0812399106.

52. Rauch TA, Zhong X, Wu X, Wang M, Kernstine KH, Wang Z, Riggs AD, Pfeifer GP. High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci U S A. 2008;105(1):252–7. doi:10.1073/pnas.0710735105.
53. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash JB, Sabunciyan S, Feinberg AP. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet. 2009;41(2):178–186. doi:10.1038/ng.298.
54. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. 2009;462(7271):315–22. doi:10.1038/nature08514.
55. Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B, Feinberg AP. Comprehensive high-throughput arrays for relative methylation (CHARM). Genome Res. 2008;18(5):780–90.
56. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A. High-throughput bisulfite sequencing in mammalian genomes. Methods (San Diego). Calif. 2009;48(3):226–32. doi:10.1016/j.ymeth.2009.05.003.
57. Dong SM, Sun DI, Benoit NE, Kuzmin I, Lerman MI, Sidransky D. Epigenetic inactivation of RASSF1A in head and neck cancer. Clin Cancer Res. 2003;9(10 Pt 1):3635–40.
58. Richards KL, Zhang B, Baggerly KA, Colella S, Lang JC, Schuller DE, Krahe R. Genome-wide hypomethylation in head and neck cancer is more pronounced in HPV-negative tumors and is associated with genomic instability. PloS One 2009;4(3):e4941.
59. Braakhuis BJ, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. Head Neck. 2002;24(2):198–206
60. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403(6772):901–6. doi:10.1038/35002607.
61. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell. 2006;11(4):441–50. doi:10.1016/j.devcel.2006.09.009.
62. Mendell JT. MicroRNAs: critical regulators of development, cellular physiology and malignancy. Cell Cycle. 2005;4(9):1179–84.
63. van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. Nat Rev Cancer. 2011;11(9):644–56. doi:10.1038/nrc3107.
64. Babu JM, Prathibha R, Jijith VS, Hariharan R, Pillai MR. A miR-centric view of head and neck cancers. Biochim Biophys Acta. 2011;1816(1):67–72. doi:10.1016/j.bbcan.2011.04.003.
65. Nohata N, Hanazawa T, Kinoshita T, Okamoto Y, Seki N. MicroRNAs function as tumor suppressors or oncogenes: Aberrant expression of microRNAs in head and neck squamous cell carcinoma. Auris Nasus Larynx. 2013;40(2):143–9 doi:10.1016/j.anl.2012.07.001
66. Chang SS, Jiang WW, Smith I, Poeta LM, Begum S, Glazer C, Shan S, Westra W, Sidransky D, Califano JA. MicroRNA alterations in head and neck squamous cell carcinoma. Int J Cancer. 2008;123(12):2791–7. doi:10.1002/ijc.23831.
67. Childs G, Fazzari M, Kung G, Kawachi N, Brandwein-Gensler M, McLemore M, Chen Q, Burk RD, Smith RV, Prystowsky MB, Belbin TJ, Schlecht NF. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol. 2009;174(3):736–45. doi:10.2353/ajpath.2009.080731.
68. Avissar M, Christensen BC, Kelsey KT, Marsit CJ. MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2009;15(8):2850–5. doi:10.1158/1078-0432.CCR-08-3131.
69. Hui AB, Lenarduzzi M, Krushel T, Waldron L, Pintilie M, Shi W, Perez-Ordonez B, Jurisica I, O'Sullivan B, Waldron J, Gullane P, Cummings B, Liu FF. Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clinical cancer research: an official journal of the

American Association for. Cancer Res. 2010;16(4):1129–39. doi:10.1158/1078-0432.CCR-09-2166.
70. Gee HE, Camps C, Buffa FM, Patiar S, Winter SC, Betts G, Homer J, Corbridge R, Cox G, West CM, Ragoussis J, Harris AL. hsa-mir-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer. 2010;116(9):2148–58. doi:10.1002/cncr.25009.
71. Winter SC, Buffa FM, Silva P, Miller C, Valentine HR, Turley H, Shah KA, Cox GJ, Corbridge RJ, Homer JJ, Musgrove B, Slevin N, Sloan P, Price P, West CM, Harris AL. Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. Cancer Res. 2007;67(7):3441–9. doi:10.1158/0008-5472.CAN-06-3322.
72. Liu CJ, Tsai MM, Hung PS, Kao SY, Liu TY, Wu KJ, Chiou SH, Lin SC, Chang KW. miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma. Cancer Res. 2010;70(4):1635–44. doi:10.1158/0008-5472.CAN-09-2291.
73. Lo WL, Yu CC, Chiou GY, Chen YW, Huang PI, Chien CS, Tseng LM, Chu PY, Lu KH, Chang KW, Kao SY, Chiou SH. MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells. J Pathol. 2011;223(4):482–95. doi:10.1002/path.2826.
74. Nohata N, Hanazawa T, Kikkawa N, Mutallip M, Sakurai D, Fujimura L, Kawakami K, Chiyomaru T, Yoshino H, Enokida H, Nakagawa M, Okamoto Y, Seki N. Tumor suppressive microRNA-375 regulates oncogene AEG-1/MTDH in head and neck squamous cell carcinoma (HNSCC). J Hum Genet. 2011;56(8):595–601. doi:10.1038/jhg.2011.66.
75. Chen Z, Jin Y, Yu D, Wang A, Mahjabeen I, Wang C, Liu X, Zhou X. Down-regulation of the microRNA-99 family members in head and neck squamous cell carcinoma. Oral Oncol. 2012;48(8):686–91. doi:10.1016/j.oraloncology.2012.02.020.
76. Lajer CB, Garnaes E, Friis-Hansen L, Norrild B, Therkildsen MH, Glud M, Rossing M, Lajer H, Svane D, Skotte L, Specht L, Buchwald C, Nielsen FC. The role of miRNAs in human papilloma virus (HPV)-associated cancers: bridging between HPV-related head and neck cancer and cervical cancer. Br J Cancer. 2012;106(9):1526–34. doi:10.1038/bjc.2012.109.
77. Hennessey PT, Sanford T, Choudhary A, Mydlarz WW, Brown D, Adai AT, Ochs MF, Ahrendt SA, Mambo E, Califano JA. Serum microRNA biomarkers for detection of non-small cell lung cancer. PloS One. 2012;7(2):e32307. doi:10.1371/journal.pone.0032307.
78. Minor J, Wang X, Zhang F, Song J, Jimeno A, Wang XJ, Lu X, Gross N, Kulesz-Martin M, Wang D, Lu SL. Methylation of microRNA-9 is a specific and sensitive biomarker for oral and oropharyngeal squamous cell carcinomas. Oral Oncology. 2012;48(1):73–8. doi:10.-1016/j.oraloncology.2011.11.006.
79. Okumura N, Yoshida H, Kitagishi Y, Nishimura Y, Matsuda S. Alternative splicings on p53, BRCA1 and PTEN genes involved in breast cancer. Biochem Biophys Res Commun. 2011;413(3):395–9. doi:10.1016/j.bbrc.2011.08.098.
80. Luco RF, Pan Q, Tominaga K, Blencowe BJ, Pereira-Smith OM, Misteli T. Regulation of alternative splicing by histone modifications. Science. 2010;327(5968):996–1000. doi:10.1126/science.1184208.
81. Herold-Mende C, Seiter S, Born AI, Patzelt E, Schupp M, Zoller J, Bosch FX, Zoller M. Expression of CD44 splice variants in squamous epithelia and squamous cell carcinomas of the head and neck. J Pathol. 1996;179(1):66–73. doi:10.1002/(SICI)1096-9896(199605)179:1 < 66::AID-PATH544 > 3.0.CO;2-5
82. Assimakopoulos D, Kolettas E, Patrikakos G, Evangelou A. The role of CD44 in the development and prognosis of head and neck squamous cell carcinomas. Histol Histopathol. 2002;17(4):1269–81.
83. Marsit CJ, Black CC, Posner MR, Kelsey KT. A genotype-phenotype examination of cyclin D1 on risk and outcome of squamous cell carcinoma of the head and neck. Clinical cancer research: an official journal of the American Association for. Cancer Res. 2008;14(8):2371–7. doi:10.1158/1078-0432.CCR-07-4368.
84. Buch S, Zhu B, Davis AG, Odom D, Siegfried JM, Grandis JR, Romkes M. Association of polymorphisms in the cyclin D1 and XPD genes and susceptibility to cancers of the upper aero-digestive tract. Mol Carcinog. 2005;42(4):222–8. doi:10.1002/mc.20086.

85. Moller-Levet CS, Betts GN, Harris AL, Homer JJ, West CM, Miller CJ. Exon array analysis of head and neck cancers identifies a hypoxia related splice variant of LAMA3 associated with a poor prognosis. PLOS Comput Biol. 2009;5(11):e1000571. doi:10.1371/journal.pcbi.1000571.
86. Cai C, Bottcher MC, Werner JA, Mandic R. Differential expression of VEGF121, VEGF165 and VEGF189 in angiomas and squamous cell carcinoma cell lines of the head and neck. Anticancer Res. 2010;30(3):805–10.
87. Mao L, Fan YH, Lotan R, Hong WK. Frequent abnormalities of FHIT, a candidate tumor suppressor gene, in head and neck cancer cell lines. Cancer Res. 1996;56(22):5128–31.

Chapter 17
Projections: Novel Therapies for HPV-Negative Cancers of the Head and Neck

Barbara Burtness

Abstract Outcomes for patients with locally advanced head and neck cancer that is not associated with human papillomavirus (HPV) have not improved substantially despite intensification of therapy and advances in surgical and radiation technique. Differences in biomolecular profile between HPV-associated and non-associated cancers provide a potential set of molecular targets for novel therapies in HPV-negative head and neck cancer. These include ERBB/HER family members, the tyrosine kinase growth factor receptor c-Met, and signaling intermediaries such as Aurora A kinase, Hedgehog, phosphoinositide 3-kinase (PI3K), and mTOR. Immune suppression has been identified in HPV-negative larynx cancers, opening up the promise of CTLA-4 and PD-1 inhibitors for head and neck cancer. Current trials are reviewed.

Keywords Head and neck cancer · Hypoxia · EGFR · Aurora kinase · c-Met · Hedgehog

17.1 Introduction

The recognition that head and neck mucosal cancers arise as two diseases, one resulting from habitual exposures such as tobacco and the other from transforming human papillomavirus infection, with distinct molecular profiles and different treatment responsiveness, has led to a reassessment of progress in the treatment of these diseases. Non-smokers with small, regionally advanced human papillomavirus (HPV)-related oropharynx cancers can expect high cure rates with standard therapy, and the most important question for these patients may be whether treatment deintensification or appropriately selected novel therapies could preserve high cure rates while minimizing toxicity and late effects of treatment. On the other hand, it has become clear that locally advanced non-HPV-related head and neck cancer (HNC) continues to carry quite a poor prognosis. A better understanding of the biology of these cancers is

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urgently needed to direct our research towards novel targets for HPV-negative HNC. This chapter reviews current trials of novel agents which may improve treatment results in this difficult disease (Table 17.1).

17.2 Treatment Outcomes for HPV-Negative Head and Neck Cancer

Complete resection is associated with high cure rates for early stage squamous cell carcinomas of the head and neck (SCCHNs), with second primary cancers including non-small cell lung cancers (NSCLCs) constituting a significant source of mortality in these patients, and high-risk p53 genetic polymorphisms conferring a hazard ratio (HR) of 3 for second malignancy [1]. For locally advanced HNCs, resection has been supplanted by chemoradiation as an organ-sparing approach in certain anatomic subsites, such as the oropharynx and larynx. As will be described below, the emergence of natural orifice surgical techniques may lead to an increased role for surgery in the management of some oropharynx cancers. HNC has historically been tightly associated with habitual exposures, such as to tobacco, areca nut, alcohol, and mate [2], [3], but in recent decades a sharp and continuing increase has been noted in oropharynx cancers related instead to transforming HPV infection, reviewed in Chapters 2 and 3 [4]. Substantial evidence exists that HPV-associated cancers have a more favorable prognosis than HNCs arising from tobacco or other carcinogenic exposures. Improvements in outcome which have previously been attributed to treatment intensification may actually have their origin in the changing prevalence of this treatment-responsive type of HNC. A corollary of this finding is that outcomes may not have improved substantially for HPV-negative cancers, and indeed 3-year survival for HPV-negative oropharynx cancer in a recent cooperative group study was 57.1 % [5]. Thus, ongoing efforts are needed to explicate the biology of HPV-negative HNC and to identify novel therapeutic strategies, whether through intensification and combination of existing therapies, or uncovering novel targets.

An inverse relationship has been described between HPV-association and mutational burden, including mutation of p53 [6], [7]. Recent whole exome sequencing of HNCs reported by two independent groups, and conducted on predominantly HPV-negative HNCs, confirmed p53 mutation as one of the most common genomic abnormalities in HNCs (cf. Chapter 15) [7], [8]. p53 mutation predicts inferior prognosis and treatment response with conventional therapies, and this effect is the strongest for p53 mutations which are truncating or which disrupt the DNA-binding domain (cf. Chapter 12 [9], [10]. Recent data imply that p53 mutation may also negatively impact response to PI3K/mTOR inhibition [11].

Many years of research in the treatment of locally advanced HNC have investigated strategies to intensify therapy. These have included increased duration of therapy via multidrug induction chemotherapy, addition of biologic agents during chemoradiation, intensity modulated and altered fractionation in the delivery of external beam radiation, or the use of maintenance therapy following completion of

Table 17.1 Current trials with novel targets in HPV-negative, poor prognosis, or unselected head and neck cancer patients

	Target	Drug	Phase of study	Identifier
Receptor tyrosine kinases				
	EGFR	Radiation with or without cetuximab	Postoperative Phase III	R0920, NCT01311063
		Cisplatin and radiation with or without nimotuzumab	Postoperative Phase III	NCT00957086
		Cetuximab and erlotinib with chemotherapy	Recurrent/metastatic, Phase II	NCT01316757
	Dual or pan-HER	Cisplatin and radiation with or without lapatinib	Definitive chemoradiation, randomized Phase II	NCT00490061
		Afatinib vs. methotrexate	Recurrent/metastatic disease, Phase III	NCT01345669
		Afatinib vs. placebo	Minimal residual disease Phase III	NCT01345682
	c-MET	LY2801653	Phase I	NCT01285037
		Cetuximab with or without tivantinib	Recurrent/metastatic, randomized Phase II	NCT01696955
Mitotic regulator				
	Aurora A kinase	Alisertib with cetuximab and radiation	Locally advanced Phase I	NCT01540682
JAK/STAT pathway				
	STAT3	STAT3 decoy	Phase 0	NCT00696176
PI3K pathway				
	mTOR	Everolimus vs. placebo	Minimal residual disease Phase III	NCT01111058
	PI3K α	BYL719 and cetuximab	Recurrent/metastatic, Phase Ib/II	NCT01602315
	Class I PI3K	BKM120	Metastatic, Phase II	NCT01737450
Hedgehog pathway				
	SMO	Saridegib (IPI-926)	Metastatic, Phase I	NCT01255800
Immune checkpoints				
	CTLA-4	Ipilimumab with cetuximab and radiation	Locally advanced, Phase I	NCT01860430
	PD-L1	MK3475	Recurrent/metastatic Phase Ib	NCT01848834
	Regulatory T cells	Metronomic low dose cyclophosphamide	Advanced disease, Phase II	NCT01581970

chemoradiation. Although early trials of induction chemotherapy were inconclusive, meta-analysis has suggested that cisplatin-based induction chemotherapy might be associated with a 2 % improvement in overall survival [12]. Two Phase III trials of cisplatin-fluorouracil vs. docetaxel-cisplatin-fluorouracil induction chemotherapy demonstrated that docetaxel-containing induction chemotherapy is superior to cisplatin-fluorouracil doublet induction therapy [13], [14]. In one European trial, the doses of chemotherapy were cisplatin 75 mg/m^2, docetaxel 75 mg/m^2, and 5-fluorouracil 750 mg/m^2 of body surface area per day for 5 days, and radiation was given as either conventional or altered fractionation without concurrent chemotherapy. Time to treatment failure and overall survival were significantly improved [13].

A parallel study utilized a more intensive induction regimen followed by concurrent chemoradiation. Given the potential for nephrotoxicity at higher cumulative cisplatin doses, the radiation sensitizer used in this trial was carboplatin rather than cisplatin. This trial also reported significant improvement in progression-free survival and overall survival for patients treated with induction chemotherapy [14]. Subsequent analysis of the oropharynx cancer cases in the latter study was undertaken to determine whether the benefit of induction differed by HPV status [15]. HPV association was determined by polymerase chain reaction (PCR) for DNA for HPV 16 and 18 viral oncoproteins E6 and E7. Material from 111 of the 264 oropharynx cancer cases in the trial was assayed for HPV association. The median overall survival for patients with non-HPV-associated oropharynx cancer was 21 months (95 % CI 13–49 months), while—with 7 years of follow up—median survival had not been reached in the HPV-associated oropharynx cancers group, for an 80 % reduction in mortality (HR = 0.2, 95 % CI 0.10–0.38; $P < 0.0001$). Subsets were small, and no significant differences emerged between the treatment arms based on HPV association.

Neither of these studies definitively established that induction therapy followed by radiation or by carboplatin concurrent with radiation was superior to the standard therapy of conventionally fractionated radiation and concurrent high dose cisplatin, as induction chemotherapy was employed in both the experimental and the control arm of each study. Accordingly, two randomized trials were initiated to compare sequential therapy of docetaxel-cisplatin-fluorouracil induction therapy followed by chemoradiation, with chemoradiation in the absence of an induction regimen. The PARADIGM trial utilized a standard approach of 70 Gy conventionally fractionated radiation with concurrent cisplatin 100 mg/m^2 of body surface area on days 1, 22, and 43 for the control arm [16]. In the experimental arm, all patients were to receive three cycles of docetaxel-cisplatin-fluorouracil induction. Those patients who responded were to receive 70 Gy of conventionally fractionated radiation concurrent with weekly carboplatin. Those patients who did not attain at least a partial response, had > 2 cm of residual neck adenopathy, or who did not complete 3 cycles of induction therapy received an alternate chemoradiation regimen with docetaxel.

The PARADIGM trial was designed before the prevalence of HPV-associated oropharynx cancer was appreciated, and before it was well understood that HPV-associated cancers display significantly better treatment response than non-HPV-associated cancers. Thus, the statistical assumptions were based on the expectation

of 55 % 3-year progression-free survival in the control arm, while the control arm 3-year progression-free survival ultimately observed was 67 %. Additionally, the trial was closed prematurely because of low accrual, after 145 of the initially proposed 330 patients were enrolled. This trial showed no improvement in progression-free or overall survival, giving no support to the hypothesis that induction chemotherapy improves progression-free or overall survival in unselected patients with locally advanced SCCHN.

The DeCIDE trial utilized a non-cisplatin-containing chemoradiation schema previously tested by investigators at the University of Chicago [17]. Patients received radiation 1.5 Gy twice daily 1 week on and 1 week off with concurrent docetaxel, hydroxyurea, and fluorouracil, for 7 weeks of therapy. Those patients randomized to the experimental arm received two cycles of docetaxel, cisplatin, and fluorouracil. This trial utilized similar statistical assumptions to those of the PARADIGM trial, and similarly closed prematurely because of low accrual. It also failed to demonstrate an improvement in progression-free or overall survival, although a subset analysis in patients with N2c or N3 disease suggested a non-significant benefit. Thus, intensification of definitive chemoradiation with the use of induction chemotherapy containing cisplatin and docetaxel has not been shown to improve survival in unselected patients, and alternate strategies for intensification of therapy for advanced HPV-negative cancers are now under investigation.

Novel approaches to treatment intensification can be envisioned which would build on our ability to profile tumor biology with the use of validated biomarkers. As discussed above and in Chapter 12, the presence of disruptive p53 mutations is associated with higher rates of recurrence for completely resected HNCs. A strategy is currently in development to intensify postoperative therapy for these patients, even when the conventional clinical risk factors of positive margin or lymph node metastasis with extracapsular extension are not present; this is currently being tested with cetuximab (R0920, NCT01311063) and a study comparing radiation to cisplatin plus radiation in the population with disruptive p53 mutation has been proposed.

Given the evidence that non-HPV-associated cancers display greater treatment resistance to both radiation and chemotherapy, one novel approach to treatment intensification is the addition of surgical resection in settings previously managed with organ-preserving chemoradiation regimens. In the oropharynx, chemoradiation has been the standard approach for locally advanced tumors since the Eastern Cooperative Oncology Group (ECOG) trial for unresectable disease permitted inclusion of patients with base of tongue cancer which met the following criteria: "base of tongue: the tumor must extend into the root of tongue, or the patient must refuse a recommended total glossectomy [18]." That trial demonstrated a survival advantage for the addition of high-dose cisplatin to radiation, when compared to radiation alone. At the time the ECOG study was conducted, surgical approaches to oropharynx cancer commonly split the mandible or involved a total glossectomy, and entailed considerable morbidity. The recent emergence of natural orifice surgical techniques utilizing either robotics or laser has led to a reexamination of primary surgery for patients with oropharynx cancer. Single institution series demonstrate the feasibility of this approach, as well as low locoregional recurrence rates in HPV-negative as well as

HPV-associated cancers [19], [20]. These findings have raised the possibility that the inclusion of transoral resection in a multimodality treatment program could improve cure rates for locally advanced HPV-negative oropharyngeal cancer. The Radiation Therapy Oncology Group has developed a randomized Phase II trial which compares standard therapy with transoral resection followed by chemoradiation for oropharynx cancers which do not stain for p16.

17.3 Hypoxia and HPV-Negative Head and Neck Cancer

As reviewed in greater depth in Chapter 14, low tissue oxygenation is present in some HNCs, and predicts for resistance to radiation or chemoradiation. Hypoxia-inducible factors (HIFs) interact with hypoxia response elements on a large number of target genes [21], [22]. Hypoxia may also be associated with a more aggressive cancer phenotype [23]. Hypoxia-inducible factor 2 (HIF2) activates epidermal growth factor receptor (EGFR)-dependent signaling via Akt and phospholipase C gamma, potentiating head and neck carcinoma cell migration and survival [24]. Among many approaches tested clinically in the attempt to improve tumor oxygenation have been the hypoxic radiosensitizer nimorazole and analogues, erythropoiesis-stimulating agents, and hypoxia-specific cytotoxic agents such as tirapazamine [25]. A meta-analysis of 37 trials of very diverse hypoxia-targeting therapies described a promising hazard for locoregional recurrence of 0.71 ($p < 0.001$) [25].

The Tasman Radiation Oncology Group (TROG) 02.02 study was a Phase III trial of concurrent chemoradiation using a standard cisplatin and radiation schedule, randomizing patients to addition of the hypoxic cytotoxin tirapazamine or no addition of tirapazamine, with a primary endpoint of overall survival [26]. The trial failed to demonstrate an overall survival benefit from the addition of tirapazamine for the total study population; however, among a subset of oropharynx cancers analyzed for p16 expression, tirapazamine was associated with an improvement in local control among p16-negative cases only (HR 0.33, $p = .13$ in this underpowered subset analysis) [27]. Further evidence for an association between hypoxia and HPV-negative head and cancer is found in an analysis of 106 oropharynx tumors by p16 and—as an indirect marker of hypoxia—HIF1α expression [28]. HIF1α expression indicative of hypoxia was present in only 35 % of p16-expressing compared with 65 % of p16-non-expressing cases. It would appear that future study of hypoxia targeting in HPV-negative patients is warranted. Novel analogues of tirapazamine display superior tissue penetration, are more selective for hypoxic cells, and exhibit greater potency [29]; these merit clinical evaluation in trials of patients selected for hypoxic tumors and unfavorable risk.

17.4 EGFR/nuclear EGFR in HPV-Negative Head and Neck Cancer

17.4.1 Biomarkers for Patient Selection for EGFR Inhibition in Head and Neck Cancer

Cetuximab, a chimeric monoclonal antibody which targets the extracellular domain of the EGFR, is the only targeted therapy with a proven impact on survival in squamous cell cancer of the head and neck. As reviewed in Chapter 4, cetuximab has monotherapy activity in previously treated metastatic/recurrent disease, and when added to chemotherapy or radiation therapy, significantly improves survival [30], [31]. The impact is modest, with response rates of 10–12 % and a median survival for patients with metastatic/recurrent disease treated with chemotherapy and cetuximab of 10.1 months [32], [33]. These results imply that the benefit of cetuximab is confined to a subpopulation of SCCHNs, but biomarkers to predict cetuximab sensitivity have been elusive.

The initial expectation was that higher expression of EGFR would identify the cancers most dependent on EGFR signaling, and that these would be the most sensitive, just as HER2-amplified breast cancer was most sensitive to trastuzumab. However, in E5397, the first randomized trial of cisplatin with cetuximab or with placebo conducted in the first-line therapy of metastatic/recurrent HNC, this hypothesis was not confirmed; on the contrary, EGFR expression at the highest intensity and density appeared to predict for relative lack of response benefit from the addition of cetuximab to chemotherapy [34]. In contrast, immunohistochemical scoring of patients enrolled in a trial of cetuximab in NSCLC appeared to indicate greater cetuximab sensitivity at higher levels of EGFR protein expression [35]. However, when a larger set of samples was examined in a pooled analysis of Phase III data from head and neck and colorectal cancer, no association between high EGFR immunohistochemical score and outcome could be confirmed [36].

Subsequent putative biomarkers of sensitivity have been EGFR gene amplification or polysomy, the presence of the truncation variant EGFRvIII, and HPV association. EGFR content in SCCHN is extremely high relative to other tumor types, and this correlates with EGFR gene amplification as assessed by fluorescence in situ hybridization (FISH). EGFR polysomy is reported in 8–53 % of HNCs and appears the lowest in early stage oral cancers [37]–[39]. Polysomy for EGFR predicts worse outcome, yet quantitative immunohistochemistry is more predictive of outcome than FISH [39]. Licitra et al. tested tumors from patients enrolled in the Vermorken trial of platin-doublet chemotherapy with or without cetuximab; 71 % were evaluable by FISH [40]. Increased EGFR copy number was common, but high-level EGFR gene amplification occurred in only 11 % of patients. No association of EGFR copy number with outcome was identified in this trial, although a number of definitions of gene amplification were tested.

The positive benefit of inhibiting EGFR can be counteracted if downstream EGFR effectors are constitutively activated. Activation of Ras, or inactivation of phosphatase and tensin homolog (PTEN), an inhibitor of phosphoinositide 3-kinase

(PI3K), can reduce responsiveness to EGFR-targeting agents in some cancers [41]. Analysis of 67 samples from E5397 compared tumors with wild-type H-Ras plus normal expression of PTEN with cancers that were PTEN null or H-Ras mutated. Although this analysis was underpowered, a strong tendency for cetuximab benefit was seen in the PTEN-expressing/non-H-Ras mutated patients, with an HR for progression in cetuximab-treated patients of 0.56 ($p = 0.07$), whereas no such tendency was observed in the PTEN null/H-Ras mutated cancers [42].

Psyrri et al., using the widely accepted cut point of 70 % of cells expressing p16 as a surrogate for HPV association, assessed the impact of p16 expression on cetuximab responsiveness among patients enrolled in the EXTREME trial [43]. They found 41 p16-expressing and 340 p16-negative cases, as would be expected for a trial in metastatic disease, given the high cure rate for p16-positive disease. Cetuximab treatment effect was similar in p16-expressing and p16-negative cases.

Panitumumab is a second monoclonal antibody targeting the extracellular domain of EGFR [44]. Panitumumab has been incorporated with chemotherapy and radiation in a Phase I trial in SCCHN, and in this setting a clinical complete response rate of 95 % was noted [45]. The monotherapy response rate for this agent in HNC has not been defined. A randomized Phase III trial comparing doublet platin-based chemotherapy with the same doublet plus panitumumab failed to meet its overall survival endpoint of a 35 % benefit [46]. An analysis of outcome by p16 status was undertaken for 443 (67 % of the total cohort) patients. This analysis was not confined to oropharynx cancers, despite the lack of evidence that p16 expression outside the oropharynx correlates with HPV association or a difference in treatment responsiveness. The proportion of cancers p16 positive was high for a metastatic disease population at 22 %, and significant numbers of p16-positive cases were noted in atypical locations such as oral cavity and larynx; both findings may be explained by the choice of a cut point of 10 % of cells p16 positive, rather than the more conventional 70 % of cells. This analysis appeared to show that the benefit of panitumumab was confined to p16-negative patients, in contrast to the findings for cetuximab reviewed above [43]. It is not possible to determine whether the discrepant findings between these two studies are driven predominantly by the difference in methodology for determining p16 positivity, or whether there may also be significant differences between the agents with respect to the p16.

17.4.2 Novel EGFR-Directed Antibodies in Head and Neck Cancer

Panitumumab has proven activity in colorectal cancer [47], but failed to improve survival in HNC, as described. Other EGFR-directed antibodies have also been studied. Nimotuzumab is a monoclonal antibody with lower binding affinity for EGFR than cetuximab or panitumumab, thus displaying lesser skin toxicity and potentially greater tumor penetration, although no advantage in activity has been noted [48]. A randomized Phase III trial at the National Cancer Center Singapore compares standard cisplatin and postoperative radiation with postoperative nimotuzumab-cisplatin

radiation, with a primary endpoint of disease-free survival (NCT00957086). Zalutumumab is a high affinity, IgG1 monoclonal antibody to EGFR. This agent was not superior to best supportive care or methotrexate in a Phase III trial in previously treated metastatic/recurrent HNC, and is no longer in development [49]. Thus, extensive experience with altered binding affinity, whether higher or lower than cetuximab's, altered immunoglobulin backbone, and humanized antibodies have failed to demonstrate an advantage over cetuximab. A current generation of trials now attempts to overcome cetuximab resistance via inhibition of additional targets.

17.4.3 Tyrosine Kinase Inhibitors

Beyond the use of antibodies targeting the extracellular domain, EGFR may be blocked via the use of tyrosine kinase inhibitors to inhibit its intracellular autophosphorylation. Gefitinib, erlotinib, and the pan-HER inhibitor afatinib have demonstrated clinical activity in HNC, independent of the presence of activating mutations in the EGFR kinase domain [50]. Nonetheless, randomized trials have failed to confirm a benefit for gefitinib vs. chemotherapy, or for the addition of gefitinib to docetaxel chemotherapy [51], [52]. Neither gefitinib nor erlotinib improves outcomes when added to chemoradiation, although biomarker analysis suggests that in the subset of patients overexpressing IGF1R, gefitinib may be beneficial [53]–[55]. Resistance to gefitinib, erlotinib, and afatinib in preclinical models is associated with a proteomic signature reflecting activation of angiogenesis and invasion pathways [55].

Lapatinib is a dual EGFR and HER2 tyrosine kinase inhibitor, which has shown a monotherapy response rate of 17 % in previously untreated patients, and of 0 % in those with recurrent/metastatic disease, whether or not the patients were previously exposed to an EGFR inhibitor [56]–[58]. Lapatinib with and following concurrent cisplatin and radiation has been compared with cisplatin and radiation in a randomized Phase II trial, demonstrating a near doubling of progression-free survival in previously untreated patients with p16-negative disease [58]. These promising findings are being pursued with a randomized Phase II trial of chemoradiation with or without lapatinib in p16-negative locally advanced HNC, conducted by the Radiation Therapy Oncology Group Foundation (NCT00490061).

As noted above, another agent that targets multiple HER family members, in this case by binding covalently and irreversibly to the kinase domains, is afatanib. Afatinib has preclinical activity in cetuximab-resistant bladder carcinoma and clinical activity in EGFR-mutated NSCLC, HER2-expressing breast cancer, and metastatic/recurrent HNC [59]–[62]. It has been compared with cetuximab monotherapy in previously treated metastatic/recurrent HNC, demonstrating comparable activity [62]. This drug is now under study in metastatic/recurrent HNC in a Phase III trial comparing afatinib with methotrexate in previously treated patients, and as an 18-month adjuvant therapy in clinical poor risk patients (non-oropharyngeal cancers likely to be HPV negative, and SCCHNs diagnosed in smokers) who are without evidence of disease following chemoradiation (NCT01345669; NCT01345682).

17.4.4 Nuclear EGFR

The role of EGFR as a tyrosine kinase growth factor receptor, with wide-ranging effects on signaling, cell survival, migration, and angiogenesis are well described, but non-canonical functions of EGFR are also of importance in cancer progression and treatment resistance [63], [64]. Localization of EGFR to the nucleus occurs in HNC, and higher EGFR content in the nucleus portends worse outcome [65], [66]. EGFR is in the phosphorylated state when translocated to the nucleus, and amino-quinazoline inhibitors of the EGFR kinase domain, including gefitinib, lapatinib, and dasatinib, block translocation of EGFR to the nucleus, whereas cellular injury from radiation increases nuclear translocation [67]–[69]. Radiation-induced nuclear translocation of EGFR is reduced by exposure to cetuximab in HNC cell lines [70], yet increased nuclear EGFR content is also a feature of cetuximab resistance in conditioned cell lines [71]. Preclinical data demonstrate additive activity of combination receptor-directed antibody with a tyrosine kinase inhibitor in head and neck squamous cell lines [72]. A Phase II trial which is currently accruing tests the use of cetuximab plus erlotinib to achieve blockade of EGFR both as a receptor and as a nuclear tyrosine kinase, on a backbone of doublet chemotherapy (NCT01316757). Further experimental strategies include the development of an EGFR-targeted antibody linked with peptides bearing nuclear localization sequences, with preclinical evidence for nuclear localization and DNA damage in EGFR-rich breast carcinoma cell lines [73].

17.5 Resistance in the Signaling Stream

The layered signaling network which transduces EGFR activation into its cellular effects is another potential source of resistance to EGFR inhibition. Resistance to EGFR inhibition results from activating mutations within the signaling stream, most notably demonstrated in colon cancer patients with K-Ras mutations, who do not benefit from EGFR inhibition [47]; however, as discussed above, such mutations are rare in HNC, and other mechanisms of activation of signaling pathways may have greater significance [74]. High throughput screening for genes which confer synthetic lethality in the setting of EGFR inhibition has been exploited to identify potential novel targets in HNC. Astsaturov et al. conducted high throughput screening of more than 600 EGFR- interacting genes to detect synthetic lethality, on the basis of cell viability, when EGFR was inhibited with erlotinib [75]. The HPV-negative, p53-mutated cell line SCC61 and others demonstrated synthetic lethality of EGFR inhibition with BCAR1, BCAR3, and NEDD9, proteins that promote metastasis and invasive behavior in HNC and other cancers [76], [77]. Although not readily druggable, NEDD9 interacts with Aurora A kinase, and exposure to the Aurora A kinase inhibitor PHA-680632 was synergistic with erlotinib. These findings raised the possibility of dual EGFR and Aurora A kinase inhibition, although the role of Aurora A kinase in HNC was not well elucidated. Tissues from 180 resected squamous cancers of the head and neck were assayed for EGFR and Aurora A expression [78]. Elevated EGFR and Aurora A protein expression both correlated with significantly

shorter disease-free and overall survival. These data supported the development of a Phase I trial, currently recruiting, which incorporates the Aurora A kinase inhibitor alisertib with cetuximab and radiation for locally advanced HNC (NCT01540682).

The signal transducer and activator of transcription (STAT) proteins, important mediators of EGFR signaling, may also have a role in HNC therapy. STAT3 is overexpressed in HNC, and STAT3 knockdown restores cetuximab sensitivity and radiation sensitivity [79]–[80]. A Phase 0 preoperative study with a STAT3 decoy has been completed in SCCHN patients and data are anticipated (NCT00696176). Pharmacologic JAK/STAT inhibitors also have promise for treatment of HPV-negative HNC with preclinical studies with JAK/STAT inhibitor AG490 inhibiting cell proliferation, inducing G1 arrest and apoptosis, and downregulating expression of STAT3, phosphorylated STAT3, and survivin in larynx carcinoma cells [81]. Preclinical studies demonstrate that the heat shock protein (HSP) 90 inhibitor SNX5422/2112 can down regulate multiple key signaling molecules in HNC, inclusive of STAT3, based on the important role of HSP90 of stabilizing these proteins in tumor cells [82].

Mutations occur within the PI3K pathway (reviewed in Chapter 7) in 30.5 % of HNCs [83], a much higher rate than for the RAF/MEK/ERK kinase or JAK/STAT pathways. Additional epigenetic mechanisms, copy number gain, and signal transduction from overexpressed tyrosine kinase receptors may result in activation of this pathway in over 80 % of HPV-negative or HPV-positive HNCs [84]. Mammalian target of rapamycin (mTOR) inhibitors and PI3K inhibitors are active in HNC both preclinically and clinically. Ongoing trials assess the mTOR inhibitor everolimus as maintenance therapy following definitive therapy, the PI3K-α-specific inhibitor BYL719 in combination with cetuximab for recurrent or metastatic disease, and the pan-class I PI3K inhibitor BKM120 in metastatic recurrent disease (NCT01111058; NCT01602315; NCT01737450). Several prior attempts at combined EGFR and mTOR inhibition in head and neck and lung cancer have faltered due to unexpectedly high rates of fulminant infection, suggesting additive effects on innate immunity between these classes [85]. In vitro studies imply that activity of the PI3K/mTOR inhibitor PF-04691502 is greatest with intact p53 function, and p53 mutation and expression may be useful biomarkers in studies of PI3K inhibition [86].

Sonic hedgehog (Shh), Patched (Ptch), and Gli-1 are components of the Hedgehog signaling pathway which are overexpressed in oral squamous cell carcinoma, with overexpression correlated to more advanced stage and higher recurrence rate [87]. Laboratory evidence links Hedgehog signaling to resistance to EGFR inhibition, and a pilot study of cetuximab and the Hedgehog pathway inhibitor saridegib (IPI-926) is ongoing in recurrent HNC (NCT01255800) [88].

17.6 c-Met

Receptor tyrosine kinases beyond the HER family are also possible therapeutic targets in SCCHN. c-Met activates pleotropic signaling pathways including PI3K/Akt, Ras/MEK/ERK, and focal adhesion kinase (FAK) [89], and appears to play a critical role in HPV-negative SCCHN. Hepatocyte growth factor (HGF or scatter factor), the

ligand of c-Met, and c-Met are present in oral cancers, and their elevated expression is correlated with higher tumor and nodal stage [90]. c-Met overexpression in 80 % of head and neck squamous cancers, MET gene amplification in 13 % of tumors, as well as lower frequency of mutation in the ligand-binding, juxtamembrane, and kinase domains are described in head and neck tumor samples and cell lines [91]. Both single agent therapy with a multikinase inhibitor with activity against c-MET, and—given the association of MET expression with cetuximab resistance, and convergent MET and EGFR signaling via the PI3K pathway—the combination of a putative c-MET inhibitor with cetuximab are currently in trial in patients with recurrent or metastatic HNC (NCT01285037; NCT01696955).

17.7 Immune Checkpoint Inhibitors

An important new direction in anticancer therapy is the targeting of immune checkpoints mediated by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152), a negative regulator of T-lymphocyte activation, and by the programmed death-1 (PD-1) receptor and its ligand (PD-L1). The CTLA-4 inhibitor ipilimumab and the PD-1 inhibitors nivolumab and MK3475 and their combinations have shown impressive activity in melanoma and NSCLC [92], [93]. CTLA-4 has a central role in immune tolerance and anergy, and across geographic regions, CTLA-4 gene polymorphisms influence risk for oral squamous carcinoma [94]–[96].

Although significant interest has been directed to the role of immune resistance in the development of HPV-associated HNC [97], evidence has now emerged for immune resistance in HPV-negative HNCs as well. CTLA-4 expressing regulatory T cells are increased in larynx cancer [98]. An increased frequency of CD8 + PD1 + is observed in the peripheral blood mononuclear cells of oral squamous cell cancer patients when compared with controls; additionally, increases in both CD4 + and CD8 + cells expressing PD-1(+) were found to accumulate in oral squamous cancers, with intense expression of PD-L1 [99]. Expression of PDL-1 has been demonstrated in a series of 238 cases of larynx cancer with high PD-L1 expression associated with favorable overall survival ($P = 0.029$) [100]. Trials are currently ongoing which assess the safety and efficacy of immune checkpoint inhibitors in HNCs. A Phase Ib trial of concurrent cetuximab and radiotherapy with the CTLA4 antagonist ipilimumab in locally advanced disease is actively recruiting (NCT01860430). A current multidisease Phase Ib study of the novel anti-PDL-1 antibody MK3475 includes squamous cell cancers of the head and neck, aiming to enroll both HPV-negative and -positive cases (NCT01848834). An alternate strategy is the use of metronomic (frequent but low dose) cyclophosphamide to deplete regulatory T cells, currently being tested in combination with cetuximab in advanced HNC (NCT01581970).

17.8 Concluding Statement

Locally advanced SCCHN remains difficult to treat, with high recurrence rates despite attempts at treatment intensification. Complete genome sequencing of HNCs and multiple platform characterization of HNC confirm the centrality of the PI3K

pathway and the receptor tyrosine kinases which activate it, in the pathogenesis of this disease. Numerous novel agents that target redundant receptor tyrosine kinases, the PI3K/mTOR pathway, and Aurora kinases are underway. In addition, the recognition of the role of immune checkpoints in some HNCs has opened the possibility of immune checkpoint inhibition in the future.

References

1. Jin L, et al. Genetic variants in p53-related genes confer susceptibility to second primary malignancy in patients with index squamous cell carcinoma of head and neck. Carcinogenesis. 2013;34:1551–7.
2. Goldenberg D, et al. Habitual risk factors for head and neck cancer. Otolaryngol Head Neck Surg. 2004;131:986–93.
3. Goldenberg D, et al. The beverage maté: a risk factor for cancer of the head and neck. Head Neck. 2003;25:595–601.
4. Schwartz SM, et al. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. J Natl Cancer Inst. 1998;90:1626–36.
5. Ang KK, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010;363:24–35.
6. Westra WH, et al. Inverse relationship between human papillomavirus-16 infection and disruptive p53 gene mutations in squamous cell carcinoma of the head and neck. Clin Cancer Res. 2008;14:366–9.
7. Stransky N, et al. The mutational landscape of head and neck squamous cell carcinoma. Science. 2011;333:1157–60.
8. Agrawal N, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science. 2011;333:1154–7.
9. Poeta ML, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med. 2007;357:2552–61.
10. Lindenbergh-van der Plas M. Prognostic significance of truncating TP53 mutations in head and neck squamous cell carcinoma. Clin Cancer Res. 2011;17:3733–41.
11. Herzog A, et al. PI3K/mTOR Inhibitor PF-04691502 antitumor activity is enhanced with induction of wild-type TP53 in human xenograft and murine knockout models of head and neck cancer. Clin Cancer Res. 2013;19:3808–19.
12. Pignon JP, et al. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol. 2009;92:4–14.
13. Vermorken JB, et al. Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. N Engl J Med. 2007;357:1695–704.
14. Posner MR, et al. Cisplatin and Fluorouracil Alone or with Docetaxel in Head and Neck Cancer. N Engl J Med. 2007;357:1705–15.
15. Posner MR, et al. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. Ann Oncol. 2011;22:1071–7.
16. Haddad R, et al. Induction chemotherapy followed by concurrent chemoradiotherapy (sequential chemoradiotherapy) versus concurrent chemoradiotherapy alone in locally advanced head and neck cancer (PARADIGM): a randomised phase 3 trial. Lancet Oncol. 2013;14:257–64.
17. Cohen EEW, et al. DeCIDE: A phase III randomized trial of docetaxel(D), cisplatin(P), 5-fluorouracil(F)(TPF) induction chemotherapy (IC) in patients with N2/N3 locally advanced squamous cell carcinoma of the head and neck (SCCHN). J Clin Oncol 2012;30 (Suppl; abstr 5500).
18. Adelstein DJ, et al. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. J Clin Oncol. 2003;21(1):92–8.

19. Haughey BH, et al. Transoral laser microsurgery as primary treatment for advanced-stage oropharyngeal cancer: a United States multicenter study. Head Neck. 2011;33:1683–94.
20. Quon H, et al. Transoral robotic surgery and adjuvant therapy for oropharyngeal carcinomas and the influence of p16(INK4a) on treatment outcomes. Laryngoscope. 2013;123:635–40.
21. Aebersold DM, et al. Expression of hypoxia-inducible factor-1 alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res. 2001;61: 2911–6.
22. Le QT, Denko NC, Giaccia AJ. Hypoxic gene expression and metastasis. Cancer Metastasis Rev. 2004;23:293–310.
23. Becker A, et al. Oxygenation of squamous cell carcinoma of the head and neck: comparison of primary tumors, neck node metastases, and normal tissue. Int J Radiat Oncol Biol Phys. 1998;42:35–41.
24. Wang X, Schneider A. HIF-2alpha-mediated activation of the epidermal growth factor receptor potentiates head and neck cancer cell migration in response to hypoxia. Carcinogenesis. 2010;31:1202–10.
25. Overgaard J. Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck—a systematic review and meta-analysis. Radiother Oncol. 2011;100:22–32.
26. Rischin D, et al. Tirapazamine, cisplatin, and radiation versus cisplatin and radiation for advanced squamous cell carcinoma of the head and neck (TROG 02.02, HeadSTART): a phase III trial of the Trans-Tasman Radiation Oncology Group. J Clin Oncol. 2010;28:2989–95.
27. Rischin D, et al. Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol. 2010;28: 4142–8.
28. Rahimi AS, et al. p16, Cyclin D1, and HIF-1α predict outcomes of patients with oropharyngeal squamous cell carcinoma treated with definitive intensity-modulated radiation therapy. Int J Otolaryngol. 2012;2012:685951.
29. Hicks KO et al. Pharmacokinetic/pharmacodynamic modeling identifies SN30000 and SN29751 as tirapazamine analogues with improved tissue penetration and hypoxic cell killing in tumors. Clin Cancer Res. 2010;16(20):4946–57.
30. Bonner JA et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. Lancet Oncol. 2010;11:21–8.
31. Vermorken JB et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359:1116–27.
32. Vermorken JB et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J Clin Oncol. 2007;25:2171–7.
33. Herbst RS, et al. Phase II multicenter study of the epidermal growth factor receptor antibody cetuximab and cisplatin for recurrent and refractory squamous cell carcinoma of the head and neck. J Clin Oncol. 2005;23:5578–87.
34. Burtness et al. Phase III randomized trial of cisplatin plus placebo compared with cisplatin plus cetuximab in metastatic/recurrent head and neck cancer: an Eastern Cooperative Oncology Group study. J Clin Oncol. 2005;23:8646–54.
35. Pirker R, et al. EGFR expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. Lancet Oncol. 2012;13:33–42.
36. Licitra L, et al. Predictive value of epidermal growth factor receptor expression for first-line chemotherapy plus cetuximab in patients with head and neck and colorectal cancer: analysis of data from the EXTREME and CRYSTAL studies. Eur J Cancer. 2013;49:1161–8.
37. Rössle M, et al. EGFR expression and copy number changes in low T-stage oral squamous cell carcinomas. Histopathology. 2013;63:271–8.
38. Chung CH, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol. 2006;24: 4170–6.

39. Pectasides E, et al. Comparative prognostic value of epidermal growth factor quantitative protein expression compared with FISH for head and neck squamous cell carcinoma. Clin Cancer Res. 2011;17:2947–54.
40. Licitra L, et al. Evaluation of EGFR gene copy number as a predictive biomarker for the efficacy of cetuximab in combination with chemotherapy in the first-line treatment of recurrent and/or metastatic squamous cell carcinoma of the head and neck: EXTREME study. Ann Oncol. 2011;22:1078–87.
41. De Roock W, et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann Oncol. 2008;19(3):508–15.
42. Burtness B, et al. Activity of cetuximab (C) in head and neck squamous cell carcinoma (HNSCC) patients (pts) with PTEN loss or PIK3CA mutation treated on E5397, a phase III trial of cisplatin (CDDP) with placebo (P) or C. J Clin Oncol. 2013;31:6028.
43. Psyrri A, et al. Safety and Efficacy of cisplatin plus 5-FU and cetuximab in HPV positive and HPV negative recurrent and/or metastatic squamous cell carcinoma of the head and neck (R/M) SCCHN: analysis of the phase III EXTREME trial. Ann Oncol. 2012;23(9):10180.
44. Foon KA, et al. Preclinical and clinical evaluations of ABX-EGF, a fully human anti-epidermal growth factor receptor antibody. Int J Radiat Oncol Biol Phys. 2004;58:984–90.
45. Wirth LJ, et al. Phase I dose-finding study of paclitaxel with panitumumab, carboplatin and intensity-modulated radiotherapy in patients with locally advanced squamous cell cancer of the head and neck. Ann Oncol. 2010; 21:342–7.
46. Vermorken JB, et al. 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. Lancet Oncol. 2013;14:697–710.
47. Van Cutsem E, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. J Clin Oncol. 2007;25:1658–64.
48. Berger C, et al. Nimotuzumab and cetuximab block ligand-independent EGF receptor signaling efficiently at different concentrations. J Immunother. 2011;34:550–5.
49. Machiels JP, et al. 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: an open-label, randomised phase 3 trial. Lancet Oncol. 2011;12:333–43.
50. Bontognali S, et al. Analysis of the EGFR mutation status in head and neck squamous cell carcinoma before treatment with Gefitinib. Onkologie. 2013;36:161–6.
51. Argiris A, et al. Phase III randomized, placebo-controlled trial of docetaxel with or without gefitinib in recurrent or metastatic head and neck cancer: an Eastern Cooperative Oncology Group trial. J Clin Oncol. 2013;31:1405–14.
52. Stewart JS, et al. Phase III study of gefitinib compared with intravenous methotrexate for recurrent squamous cell carcinoma of the head and neck. J Clin Oncol. 2009;27:1864–71.
53. Gregoire V, et al. Gefitinib plus cisplatin and radiotherapy in previously untreated head and neck squamous cell carcinoma: a phase II, randomized, double-blind, placebo-controlled study. Radiother Oncol. 2011;100:62–9.
54. Thariat J, et al. Contrasted outcomes to gefitinib on tumoral IGF1R expression in head and neck cancer patients receiving postoperative chemoradiation (GORTEC trial 2004–02). Clin Cancer Res. 2012;18:5123–33.
55. Box C, et al. A novel serum protein signature associated with resistance to epidermal growth factor receptor tyrosine kinase inhibitors in head and neck squamous cell carcinoma. Eur J Cancer. 2013. doi:pii:S0959-8049(13)00213-X.
56. Del Campo JM, et al. 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. Br J Cancer. 2011;105:618–27.
57. de Souza JA, et al. A phase II study of lapatinib in recurrent/metastatic squamous cell carcinoma of the head and neck. Clin Cancer Res. 2012;18:2336–43.

58. Harrington K, et al. Randomised Phase II study of oral lapatinib combined with chemoradiotherapy in patients with advanced squamous cell carcinoma of the head and neck: rationale for future randomised trials in human papilloma virus-negative disease. Eur J Cancer. 2013;49:1609–18.
59. Quesnelle KM, Grandis JR. Dual kinase inhibition of EGFR and HER2 overcomes resistance to cetuximab in a novel in vivo model of acquired cetuximab resistance. Clin Cancer Res. 2011;17:5935–44.
60. Miller VA, et al. Afatinib versus placebo for patients with advanced, metastatic non-small-cell lung cancer after failure of erlotinib, gefitinib, or both, and one or two lines of chemotherapy (LUX-Lung 1): a phase 2b/3 randomised trial. Lancet Oncol. 2012;13:528–38, Erratum in: Lancet Oncol. 2012;13:e186.
61. Lin NU, et al. A phase II study of afatinib (BIBW 2992), an irreversible ErbB family blocker, in patients with HER2-positive metastatic breast cancer progressing after trastuzumab. Breast Cancer Res Treat. 2012;133:1057–65.
62. Seiwert TY, et al. BIBW 2992 versus cetuximab in patients with metastatic or recurrent head and neck cancer (SCCHN) after failure of platinum-containing therapy with a cross-over period for progressing patients: Preliminary results of a randomized, open-label phase II study. J Clin Oncol. 2010;28:15s, 5501.
63. Shi Y, et al. Nuclear epidermal growth factor receptor interacts with transcriptional intermediary factor 2 to activate cyclin D1 gene expression triggered by the oncoprotein latent membrane protein 1. Carcinogenesis. 2012;33:1468–78.
64. Wang YN, Hung MC. Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. Cell Biosci. 2012;2:13.
65. Psyrri A, et al. Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. Clin Cancer Res. 2005;11:5856–62.
66. Psyrri A, et al. Correlates and determinants of nuclear epidermal growth factor receptor content in an oropharyngeal cancer tissue microarray. Cancer Epidemiol Biomarkers Prev 2008;17:1486–92.
67. Friedmann BJ, et al. Interaction of the epidermal growth factor receptor and the DNA-dependent protein kinase pathway following gefitinib treatment. Mol Cancer Ther. 2006;5:209–18.
68. Kim HP, et al. Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2. PLoS One. 2009;4:e5933.
69. Li C, et al. Dasatinib blocks cetuximab- and radiation-induced nuclear translocation of the epidermal growth factor receptor in head and neck squamous cell carcinoma. Radiother Oncol. 2010;97:330–7.22–24.
70. Dittmann K, et al. Inhibition of radiation-induced EGFR nuclear import by C225 (Cetuximab) suppresses DNA-PK activity. Radiother Oncol. 2005;76:157–61.
71. Li C, et al. Nuclear EGFR contributes to acquired resistance to cetuximab. Oncogene. 2009;28:3801–13.
72. Huang S, et al. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. Cancer Res. 2004;64(15):5355–62.
73. Fasih A, et al. (111)In-Bn-DTPA-nimotuzumab with/without modification with nuclear translocation sequence (NLS) peptides: an Auger electron-emitting radioimmunotherapeutic agent for EGFR-positive and trastuzumab (Herceptin)-resistant breast cancer. Breast Cancer Res Treat. 2012;135:189–200.
74. Van Cutsem E, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. J Clin Oncol. 2011;29:2011–9.
75. Astsaturov I, et al. Synthetic Lethal Screen of an EGFR-Centered Network to Improve Targeted Therapies. Sci Signal. 2010;3:67–84.

76. Tikhmyanova N, Little JL, Golemis EA. CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci. 2010;67:1025–48.
77. Lucas JT Jr, et al. Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent. Oncogene. 2010;29:4449–59.
78. Hoellein A, et al. Aurora kinase inhibition overcomes cetuximab resistance in squamous cell cancer of the head and neck. Oncotarget. 2011;2:599–609.
79. Pectasides E, et al. Nuclear localization of signal transducer and activator of transcription 3 in head and neck squamous cell carcinoma is associated with a better prognosis. Clin Cancer Res. 2010;16:2427–34.
80. Bonner JA, et al. Inhibition of STAT-3 results in greater cetuximab sensitivity in head and neck squamous cell carcinoma. Radiother Oncol. 2011;99:339–43.
81. Sen M, et al. Targeting Stat3 abrogates EGFR inhibitor resistance in cancer. Clin Cancer Res. 2012;18:4986–96.
82. Friedman JA, et al. HSP90 Inhibitor SNX5422/2112 targets the dysregulated signal and transcription factor network and malignant phenotype of head and neck squamous cell carcinoma. Transl Oncol. 2013;6:429–41.
83. Lui VWY, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer Discov. 2013;3:761–9.
84. Iglesias-Bartolome R, Martin D, Gutkind JS. Exploiting the head and neck cancer oncogenome: widespread PI3K-mTOR pathway alterations and novel molecular targets. Cancer Discov. 2013;3:722–5.
85. Burtness B, et al. Comment on “epidermal growth factor receptor is essential for toll-like receptor 3 signaling”. Sci Signal. 2012;5:lc5.
86. Herzog A, et al. PI3K/mTOR inhibitor PF-04691502 antitumor activity is enhanced with induction of wild-type TP53 in human xenograft and murine knockout models of head and neck cancer. Clin Cancer Res. 2013;19:3808–19.
87. Wang YF, et al. Expression of hedgehog signaling molecules as a prognostic indicator of oral squamous cell carcinoma. Head Neck. 2012;34:1556–61.
88. Keysar SB, et al. Hedgehog signaling alters reliance on EGF receptor signaling and mediates anti-EGFR therapeutic resistance in head and neck cancer. Cancer Res. 2013;73:3381–92.
89. Appleman LJ. MET signaling pathway: a rational target for cancer therapy. J Clin Oncol. 2011;29:4837–844.
90. Chen YS, et al. Expression of hepatocyte growth factor and c-met protein is significantly associated with the progression of oral squamous cell carcinoma in Taiwan. J Oral Pathol Med. 2004;33:209–17.45.
91. Seiwert TY, et al. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. Cancer Res. 2009;69:3021–31.
92. Hamid O, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med. 2013;369:134–44.
93. Wolchok JD, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369(2):122–33.
94. Wong YK, et al. Association of CTLA-4 gene polymorphism with oral squamous cell carcinoma. J Oral Pathol Med. 2006;35:51–4.
95. Erfani N, et al. Strong Association of CTLA-4 Variation (CT60A/G) and CTLA-4 haplotypes with predisposition of Iranians to head and neck cancer. Iran J Immunol. 2012;9:188–98.
96. Bharti V, Mohanti BK, Das SN. Functional genetic variants of CTLA-4 and risk of tobacco-related oral carcinoma in high-risk North Indian population. Hum Immunol. 2013;74:348–52.
97. Lyford-Pike S, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013;73:1733–41.
98. Erfani N, et al. Intracellular CTLA4 and regulatory T cells in patients with laryngeal squamous cell carcinoma. Immunol Invest. 2013;42:81–90.
99. Malaspina TS. et al. Enhanced programmed death 1 (PD-1) and PD-1 ligand (PD-L1) expression in patients with actinic cheilitis and oral squamous cell carcinoma. Cancer Immunol Immunother. 2011;60:965–74.
100. Vasilakopoulou M. et al. Effect of PDL-1 expression on prognosis in head and neck squamous cell carcinoma. J Clin Oncol. 2013;31:6012.

Index

β-catenin, 202–207, 217–222
TP53 tp, 247

A
Aurora Kinase, 352, 355

B
Biomarkers, 120, 121, 124, 132, 137, 142, 146–148, 327–329, 334

C
c-Met, 104, 353, 354
Cancer stem cell (CSC), 179, 188, 189, 191, 206
Cetuximab, 67–78
Chemoresistance, 237
Cisplatin, 56, 63, 68–70, 72, 73, 260, 262, 265, 266, 269–273, 275

D
Detection, 241, 244
Disruptive mutation, 242
DNA methylation, 329, 331
DNA repair, 259, 260, 262, 265, 268, 271, 275, 276
Dose de-escalation, 8, 17, 18

E
Epidermal Growth Factor Receptor (EGFR), 78, 92, 98–102, 349–353
Epigenetics, 205, 326
Epithelial-mesenchymal transition (EMT), 179, 180, 182–192
ErbB family, 56, 58, 63, 71, 72
Erlotinib, 72, 75, 77, 78

F
Fluoroazomycinarabinofuranoside (FAZA), 290
Fluoromisonidazole, 289

G
Gene expression, 325–332, 334, 335
Genomics, 317, 318

H
Head and neck cancer (HNC), 92, 93, 100, 166–168, 170–172, 179, 181–185, 188–191, 219, 220, 228, 230, 234, 242–246, 308, 315, 325, 332, 343, 344, 347–354
Head and neck squamous cell carcinoma (HNSCC), 92, 132, 133, 137, 140–143, 145–149, 283–291, 293, 294, 296, 297
Hepatocyte growth factor (HGF), 93, 96–102, 104
Hepatocyte growth factor receptor (HGFR), 92, 93, 95
Human papilloma virus (HPV), 8, 11–15, 18, 23–27, 29, 141–143, 206, 207, 215, 216, 219, 222, 229, 230, 233, 241, 284, 297, 326, 327, 332
  markers of, 220
Hypoxia, 284, 285, 287, 289–292, 294–298, 348
Hypoxia induced factor (HIF-1a), 294, 295

I
Incidence, 30, 32, 34, 36, 38
Insulin receptor substrate (IRS), 115
  Insulin receptor substrate-1 (IRS-1), 116, 118, 122
  Insulin receptor substrate-2 (IRS-2), 115
Insulin-like growth factor receptor-1 receptor (IGF1R), 118, 119
Invadopodia, 120

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J
Jak, 164, 166, 168, 172

M
Mammalian target of rapamycin (mTOR), 136, 137, 141–143, 145–148
Metastasis, 117, 119, 120, 122, 179–185, 188–191
Microarray expression studies, 219
MicroRNAs (miRs), 179, 189, 326, 332
Monoclonal antibodies, 58, 67, 71, 73
Mortality, 31

N
Nitroimidazole, 284, 289, 290, 293
Notch, 305, 310–315

O
Oncogenome, 208
Oral cancer, 206
Oropharyngeal cancer, 13, 29, 34, 36–39, 42
Oxygen enhancement ratio (OER), 286

P
Phosphatase and tensin homologue (PTEN), 134, 137, 140, 141, 146
Phosphoinositide 3-kinase (PI3K), 132, 133
Pimonidazole, 287, 289, 296
Prognosis, 229, 230, 232, 235, 240, 242

R
Radiation, 56, 66, 68, 69, 73, 260, 262, 265, 266, 270, 272, 273, 275, 276, 284, 286, 287, 291–293, 297
Radioresistance, 287, 290, 292, 294, 295, 298
Receptor tyrosine kinases (RTKs), 92

S
Sequencing, 304–308, 310, 311, 313, 318
Signal transduction, 205
Smad, 180, 181, 183–185
Snail, 179, 182, 184–187, 189, 191, 192
Sonic hedgehog (Shh), 218, 353
Squamous, 306, 310–312, 316
Squamous cell cancer, 97
Squamous cell carcinoma, 167
Squamous cell carcinoma of the head and neck (HNSCC), 200
Squamous cell carcinoma of the head and neck (SCCHN), 7, 9–11, 13–15, 18, 56–58, 61–63, 65–73, 75, 76
STAT, 164, 166–170

T
Targeted therapy, 99–101, 105, 173
Tirapazamine (TPZ), 293, 297
Transforming growth factor-beta (TGFβ), 179–181, 183–185, 187–189, 191
Tumor suppressor gene, 241
Twist, 179, 186–189, 191
Tyrosine kinase inhibitor (TKI), 71, 72

V
Variants, 23, 39–42

W
Wnt wnt, 208
Wnt pathway, 215–222
  negative regulation of, 223