

# Targeting cellular and molecular drivers of head and neck squamous cell carcinoma: current options and emerging perspectives

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**Abstract** Despite improvements in functional outcomes attributable to advances in radiotherapy, chemotherapy, surgical techniques, and imaging techniques, survival in head and neck squamous cell carcinoma (HNSCC) patients has improved only marginally during the last couple of decades, and optimal therapy has yet to be devised. Genomic complexity and intratumoral genetic heterogeneity may contribute to treatment resistance and the propensity for locoregional recurrence. Countering this, it demands a significant effort from both basic and clinical scientists in the search for more effective targeted therapies. Recent genomewide studies have provided valuable insights into the genetic basis of HNSCC, uncovering potential new therapeutic opportunities. In addition, several studies have elucidated how inflammatory, immune, and stromal cells contribute to the particular properties of these neoplasms. In the present review, we introduce recent

findings on genomic aberrations resulting from whole-genome sequencing of HNSCC, we discuss how the particular microenvironment affects the pathogenesis of this disease, and we describe clinical trials exploring new perspectives on the use of combined genetic and cellular targeted therapies.

**Keywords** Head and neck cancer · Human papillomavirus · Tumor microenvironment · Squamous cell carcinoma · Target therapy

## 1 Introduction

Cancers of the upper aerodigestive tract predominantly accounts for squamous cell carcinomas (SCCs), which develop in the epithelial linings of the oral cavity, pharynx, and larynx—the so-called head and neck SCCs (HNSCCs) [1].

HNSCC has been shown to contain unexpected complexity in terms of etiology, pathogenesis, morphological characteristics, clinical features, and natural history. The disease is strongly associated with tobacco use, heavy alcohol consumption, chewing of betel quid, and poor oral hygiene [2–4]. However, although these exposures account for the majority of cases of HNSCC globally, specific oncogenic (high-risk) types of human papillomavirus (HPV)—most frequently HPV type 16 (HPV16)—have been shown to be causally related to a subset of oropharyngeal SCCs (OPSCCs) that arise from the crypt epithelium of the palatine tonsils and the base of the tongue [5–8]. Furthermore, cases of HPV-related OPSCC have been increasing dramatically and now account for 50 % of cases in Europe and 65 % of cases in the US [9]. HPV-positive disease represents a distinct clinical and epidemiological condition that differs in terms of risk factors,

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molecular genetic alterations, microscopic appearance, and clinical behavior [10].

Treatment of patients with early-stage HNSCC is relatively successful and relies on straight single-modality therapy: either surgery and/or radiation alone. Unfortunately, at the time of diagnosis, the majority of patients present with locally advanced disease and are managed by combined modality treatment strategies that may profoundly affect quality of life [11].

Despite improvements in functional outcomes attributable to advances in radiotherapy, chemotherapy, and surgical and imaging techniques [12–14], survival in patients with HNSCC has not satisfactorily improved during the last couple of decades [15]. Genomic complexity [16], intratumoral genetic heterogeneity [17], and field cancerization [18] may contribute to its resistance to treatment and propensity for locoregional or distant recurrence. Additionally, patients with HNSCC often have limited options for reirradiation [19] or salvage surgery [20] and have only modest responses to second-line systemic therapies [21–23]. Moreover, although HPV-positive HNSCCs have better clinical outcomes and more favorable responses to radiochemotherapy, compared with HPV-negative HNSCCs [24], a subgroup of patients with HPV-positive HNSCCs experienced high rates of distant failure after concurrent chemoradiation [25], and contradictory results in terms of activity have been reported for drugs targeting the epidermal growth factor (EGF)/EGF receptor (EGFR) pathway in these patients [26–28].

The generally poor outcomes in patients with HNSCC demand a significant effort from both basic and clinical scientists in the search for more effective targeted therapies. Recent genomewide studies [16, 29–33] have provided valuable insights into the genetic basis of HNSCC, opening potential new targetable biologic pathways. In addition, several studies have elucidated how inflammatory, immune, and stromal cells contribute to the particular properties of these neoplasms [34, 35].

In the present review, we pursued the following aims: (1) to discuss the recent findings on genomic aberrations resulting from whole-genome sequencing of HNSCC, (2) to discuss how the particular tumor microenvironment affects the pathogenesis of the disease, and (3) to describe clinical trials exploring the use of combined genetic and cellular targeted therapies.

## 2 Genetic abnormalities in HNSCCs

Genome and exome analysis using advanced technical approaches [16, 29, 30, 32, 33, 36] has provided a comprehensive view of the genetic alterations in HNSCC and has underlined several significant properties of these tumors. First, HNSCC tumors have remarkable genetic heterogeneity, which appears to be significantly wider than that reported by Chung *et al.* almost 10 years ago [37]. Second, the gene

expression subtypes in HNSCC correspond to the histopathological classification of basal, mesenchymal, atypical, and classical variants and may provide a complementary classification tool for HNSCCs [38]. Third, HPV-positive and HPV-negative HNSCCs have different genetic drivers [33, 34, 36], although distinct hits may converge to generate common effects. Finally, HPV-driven tumors have less gross chromosomal aberrations and approximately half of the mutation rate of HPV-negative HNSCCs (30). These findings have not reached clinical relevance yet, and HPV-negative and HPV-positive tumors are currently treated the same way.

### 2.1 The dominant role of cell cycle and survival genes: TP53/RB pathway

The tumor suppressor genes *TP53* and *CDKN2A* and the oncogene *CCND1* represent the most commonly mutated genes in HPV-negative HNSCCs [16, 29, 30, 33, 39] and in premalignant dysplastic lesions [40]. DNA damage and oncogenic stress activate p53, which translates stress signals into cell cycle arrest or apoptosis. Three major mechanisms of p53 inactivation have been detected in HNSCC cells: (1) *TP53* somatic mutations, which do not cause loss of function but do result in atypical dominant-negative p53 mutants [41] (this condition, frequently described as a gain of function, is better specified as “subversion of function,” as proposed by Muller and Vousden [42]); (2) p53 degradation mediated by HPV E6 oncoprotein [43]; and (3) p53 proteasomal degradation, which requires binding of p53 with the negative regulator Mdm2 (Hdm2 in humans) [44].

Differentiated cells or basal cells that accumulate defective p53 protein become susceptible to other genetic mutations, acquire a propensity for metastasis [45], and create a favorable environment for the development of multiple independent transforming events in the same patient [46]. This explains why mutations of the *TP53* gene correlate with worse prognosis and higher risk of recurrence after definitive locoregional treatment [47]. *TP53* mutations in HNSCC are also associated with poor responses to chemotherapy and radioresistance [48], possibly *via* the inhibition of radiation-induced senescence [49].

Genomic studies have confirmed that other genes involved in cell cycle regulation, such as *CDKN2A* and *CCND1*, present high frequency of alterations in HPV-negative HNSCC [16, 29, 30]. Cyclin D1, the protein produced by *CCND1*, promotes cell proliferation in association with kinases CDK4 and CDK6. Extensive research led to demonstrate that the cyclin D1-CDK4/6 complex phosphorylates (activates) the retinoblastoma protein (pRB) and allows progression to mitosis. At the end of cell division, p16-INK4A (the protein produced by *CDKN2A*) blocks cyclin D1-CDK4/6 complex, inhibits pRB phosphorylation, and

arrests cell cycle in G1 [50]. In the absence of p16-INK4A, pRB is continuously phosphorylated, and cell proliferation proceeds [51]. p14-ARF (also called p16-INK4B), an alternate reading frame protein product of the *CDKN2A* gene, is also involved in cell cycle regulation. p14-ARF inhibits p53-dependent cell cycle arrest by interacting with MDM2 and inducing p53 ubiquitination [52]. p14-ARF block p53 function and deregulates cell cycle control [53]. Structural abnormalities of the *CDKN2A* gene in HNSCC patients lead to less production or loss of p14-ARF and limitless replicative potential [54]. Similarly, high levels of cyclin D1 due to amplifications in the *CCND1* gene result in an uncontrolled cell cycle and strongly predict unfavorable outcomes in patients with HNSCC [55].

Alterations in the p53 and pRB pathways are radically different in HPV-positive tumors [56], where p53 and pRB proteins are inactivated by viral oncoproteins E6 and E7, respectively. Specifically, E6 binds to the cellular protein E6AP, and the E6/E6AP complex is responsible for ubiquitination and proteasome degradation of p53. E7, on the other hand, inactivates pRB, which in turn induces overexpression of p16-INK4A, and cell G1-S phase transition [57].

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#### Outlook and clinical challenges

The search for a targeted therapy aimed to modulate p53 has been characterized by limited success, so far. Noteworthy is a single phase III study with adenoviral p53 gene therapy and methotrexate: wild-type p53 patients showed better response to gene therapy (probably related to up-regulation of MDM2), suggesting a role of p-53 profile as predictive biomarker [58].

Therapeutic strategies targeting p16 have not reached clinical trials yet, whereas cyclin D1-CDK 4/6 dual inhibitor is currently being tested in a phase I trial of various advanced cancers (ClinicalTrials.gov identifier: NCT01394016). Another phase I study of a CDK inhibitor in combination with radiotherapy has completed recruitment (ClinicalTrials.gov identifier: NCT00899054). Palbociclib, a target inhibitor of CDK4/6, recently approved for the treatment of breast cancer [59], has been evaluated in combination with cetuximab in a phase I study [60], and a randomized, multicenter, phase II study with palbociclib and cetuximab in HPV-negative recurrent/metastatic HNSCC is also ongoing (ClinicalTrials.gov identifier: NCT02499120).

Regarding HPV-positive tumors, preventive and therapeutic anti-HPV vaccines have been developed in an effort to prevent primary or established infections [61]. As prophylactic vaccines are based on L1 viral capsid protein, which is unexpressed in HPV-associated neoplasms and induces only humoral immunity, they are ineffective for established HPV-driven SCCs. Since it stimulates cytotoxic T lymphocytes (CTLs) against infected and transformed cells expressing specific E6 and E7 epitopes specific E6 and E7 epitopes [62], therapeutic vaccination is, conversely, a promising option [63]. Several phase I and II clinical trials are currently investigating the safety and efficacy of therapeutic DNA vaccines (ClinicalTrials.gov identifier: NCT01493154; NCT0216305), protein vaccines (ClinicalTrials.gov identifier: NCT00704041; NCT00257738; NCT00019110), and bacterial vector vaccines (ClinicalTrials.gov identifier: NCT02002182; NCT01598792) for HPV-positive HNSCC, alone or in combination with both chemotherapy and radiation therapy [63–65].

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## 2.2 Genes of cell growth as targets for biological therapy: PI3K/AKT/mTOR pathway

*PI3K* represents the second most important target gene across human cancers [66], and alterations of the PI3K pathway are common drivers in HNSCC [16, 29, 30, 33, 39]. A number of growth factors relay signals through the PI3K signaling cascade. Activated PI3K phosphorylates the second messenger phosphatidylinositols PIP2 and PIP3 and turns on downstream effectors AKT and mammalian target of rapamycin complex 2 (mTORC2). Fine-tuning of PI3K depends on opposing regulators. Phosphatase and tensin homologue (PTEN) shuts off PI3K signaling, whereas PI3K catalytic subunit alpha (PI3KCA) is responsible for complex activation [66]. The oncogene product RAS is also a positive regulator of the PI3K signaling cascade, resulting in cell survival and cell cycle regulation [67].

Abnormal PI3K pathways in HNSCC are derived mostly from gain-of-function mutations of *PI3KCA* and loss-of-function mutations of *PTEN* [16, 29, 30, 33, 39]. Because the global frequency of mutations affecting various components of the PI3K pathway is very high, and as multiple ligands and receptor tyrosine kinases rely on PI3K, the PI3K pathway has become an elective therapeutic target in HNSCC [16].

Being in the crossroad with RAS and PI3K, MEK, ERK-1, and ERK-2 are also object of several translational studies.

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#### Outlook and clinical challenges

The three major classes of PI3K inhibitors—namely, combined PI3K/mTORC, Pan-Class I, and alpha-specific—are currently under clinical evaluation in phase I and II studies, alone or in combination with either chemotherapy or cetuximab (ClinicalTrials.gov identifier: NCT00854152; NCT01737450; and NCT01252628). In particular, some controlled trials employing agents that target PI3K isoforms in recurrent/metastatic setting are worthy of mention. Two phase II trials failed to demonstrate benefit when PX-866 (Oncothyreon, Seattle, WA) was added to either docetaxel or cetuximab. In a phase I trial, BYL 719 (Novartis Pharmaceuticals), which targets alpha isoform of class I PI3K, gave only a partial response, and further investigations are ongoing to ascertain its clinical benefit [68–70].

The mTORC inhibitors everolimus and temsirolimus, which are currently used to treat breast cancer, renal cell carcinoma, and pancreatic neuroendocrine tumors, have been also evaluated in combination with erlotinib in platinum-refractory, recurrent/metastatic HNSCC [71]. Both agents showed modest response rate and low tolerability, raising some concern in targeting the EGFR and mTOR pathways together [72, 73]. Temsirolimus has been tested as single agent in the same setting: although no objective response was recorded, a progression-free survival (PFS) rate of 40 % at 12 weeks has been achieved [74].

Other inhibitors of tyrosine kinase are also under investigation, such as trametinib, a MEK inhibitor used in combination with AKT inhibitors (ClinicalTrials.gov identifier: NCT01725100), and sorafenib, a multiple tyrosine kinase inhibitor. In a phase II study, treatment with sorafenib has shown poor response rate, but compared favorably with other phase II single agent trials in terms of progression-free and overall survival [75]. Moreover, *in vitro* experiments indicate that sorafenib might sensitize head and neck squamous cells to ionizing radiation, suggesting the potential to overcome radioresistance mainly through the inhibition of DNA double-strand breaks (DSB) [76].

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### 2.3 EGFR pathway

PI3K signaling is initiated by specific growth factors and coupled receptors, such as the EGF/EGFR.

EGFR is part of the ERB family of receptor tyrosine kinases, which includes also ERBB2, ERBB3, and ERBB4. EGF/EGFR complex activates a number of biological functions through downstream PI3K/AKT, Ras/Raf/MAPK, and JAK/STAT. It is also able to translocate to the nucleus and activate transcription, thus producing pleiotropic effects in cellular homeostasis. One of the genes induced by intranuclear EGF/EGFR is the aforementioned *CCND1* [77].

*EGFR* genetic alterations include amplifications and gain-of-function mutations that induce high protein overexpression in a large proportion of HNSCCs and lead to tumor proliferation, angiogenesis, metastasis, and consequently poor prognosis of the disease [77, 78]. However, EGFR overexpression has not been found to be a predictive biomarker of activity with EGFR-targeted therapies [79].

#### Outlook and clinical challenges

Inhibition of the EGF/EGFR pathway has been the first molecular strategy showing significant pro-survival effect in HNSCC. Inhibitors include recombinant-chimeric (cetuximab) or humanized (nimotuzumab) or fully human (panitumumab and zalutumumab) anti-EGFR monoclonal antibodies. Several controlled clinical studies have confirmed the efficacy of cetuximab in both locally advanced disease (in combination with radiotherapy) and metastatic or recurrent HNSCC (in combination with standard chemotherapy) [14, 21].

In platinum-refractory or ineligible patients, cetuximab has shown modest activity as monotherapy [80], but encouraging response rate in combination with paclitaxel in a phase II trial [81]. Benefits achieved with cetuximab were not confirmed for panitumumab in patients with metastatic or recurrent disease [26]. Similarly, disappointing results in terms of overall survival were reported for zalutumumab compared with BSC alone in a phase III study in recurrent/metastatic setting. Nimotuzumab provided survival benefit in inoperable advanced Indian patients in a randomized phase IIb, 5-year study [22, 78]. Contrasting these results, inhibitors of tyrosine kinase activity using small molecules, which block the phosphorylation and activation of EGFR (gefitinib and erlotinib), have shown limited antitumor activity [82, 83], and no additional studies have been planned.

Patients treated with EGFR inhibitors develop high levels of *de novo* or acquired resistance to therapy. This may be due to activation of other ErbB-family receptors, cross-talk with other signaling pathways, nuclear localization of EGFR, or mutant forms of the receptor [84, 85]. Therefore, interest is currently shifting to the use of inhibitors that target multiple ERB-family members. Initially, lapatinib, a reversible tyrosine kinase inhibitor of EGFR and ERBB2, has shown promising activity, when used in combination with concurrent chemoradiotherapy in HPV-negative patients [86]. However, in a large adjuvant post-operative phase III study, lapatinib added to concurrent chemoradiation and used as long-term maintenance therapy has failed to improve both disease-free and overall survival in high-risk HNSCC patients, and has caused additional toxicity compared with placebo [87]. These findings should make us reflect on the opportunity to have reliable data on the effectiveness of targeted therapies before programming large controlled studies. At present, investigation of lapatinib is restricted to a single phase II trial in the advanced setting (ClinicalTrials.gov identifier: NCT01044433). Another ERB-family blocker, afatinib, has shown a response rate similar to that of cetuximab in

a phase II randomized trial, with a lack of cross-resistance following sequential EGFR/ErbB therapy [88]. On the basis of these data, afatinib has moved to a phase III trial in recurrent/metastatic setting, confirming its efficacy compared to methotrexate (LUX Head & Neck 1 study), in terms of progression-free survival and patient-reported outcomes [23]. A new trial comparing the efficacy of afatinib with placebo as adjuvant therapy in patients who have received definitive chemoradiotherapy (LUX Head & Neck 2) is currently recruiting participants (ClinicalTrials.gov identifier: NCT01345669). Table 1 summarizes the results of the most relevant clinical trials targeting the EGF/EGFR complex.

### 2.4 Genes of squamous cell differentiation: the NOTCH pathway

One important finding of the whole-exome sequencing studies is the high frequency of mutations (up to 15 %) in *NOTCH1* gene [29, 30]. The NOTCH signaling pathway is activated when one cell expressing the appropriate ligand (Jagged or Delta) interacts with a neighbor cell expressing a NOTCH1 receptor. The NOTCH receptor is cleaved by ADAM metalloprotease and  $\gamma$ -secretase complex, and the intracellular domain translocates to the nucleus, where it activates transcription of target genes *HES1* and *HEY1* [89]. In human keratinocytes, NOTCH1 signaling is essential to promote cell differentiation, and down-modulation or loss-of-function mutations of *NOTCH1* gene are associated with dysfunctional squamous cell differentiation and development of carcinoma [90]. Fine-tuning of NOTCH signaling depends on a number of regulators. Relevant for cancer development is the reciprocal feedback loop between NOTCH, p53, and p63, which contributes to the balance between self-renewing and differentiation of keratinocytes. Suppression of p53 activity down-regulates *NOTCH1*, blocks differentiation, and promotes uncontrolled cell proliferation [91]. High levels of p63 also inhibit NOTCH1 and suppress differentiation, whereas low levels of p63 and high levels of NOTCH1 result in the opposite effect [92].

*NOTCH1* mutations have been detected in a large proportion of HNSCCs, making *NOTCH1* the second most frequently mutated gene after *TP53* in these tumors [29, 30, 39]. Several mutations result in *NOTCH1* inactivation, suggesting a tumor suppressor function rather than an oncogene function. Only a small subset of patients with HNSCC present with gain-of-function mutations [29, 30, 93], which are similar to those associated with the leukemia cluster [94]. Mutations of other genes of the NOTCH1 pathway, in the presence of wild-type *NOTCH*, have also been detected in patients with HNSCC [93].

#### Outlook and clinical challenges

The NOTCH1 pathway represents a potential new target in cancer therapy, although a therapeutic approach is complicated by the dual nature of tumor suppressor and oncogene of *NOTCH1*. There are currently no available targeted drugs for this pathway. Inhibitors or activators of the NOTCH1 pathway *via* block of  $\gamma$ -secretase and histone deacetylase, respectively, are developing.

**Table 1** Relevant controlled clinical trials targeting EGF/EGFR pathways

Agent	Phase II–III trials	Clinical setting	Main results	Reference(s)
Cetuximab (chimeric human anti-EGFR)	Phase III plus RT	Locally advanced	Improved OS	Bonner <i>et al.</i> 2010 [8]
Panitumumab (fully human anti-EGFR mAb)	Phase III plus CT (extreme spectrum)	Recurrent/metastatic	Improved OS	Vermorken <i>et al.</i> 2008 [15]
Nimotuzumab (humanized anti-EGFR mAb)	Phase III plus CT	Recurrent/metastatic	Negative study, but improved OS in post-hoc analysis in HPV negative	Vermorken <i>et al.</i> 2013 [20]
Zalutumumab (fully human anti-EGFR mAb)	Phase IIb plus CRT or RT	Locally advanced	Improved survival (median not reached for nimotuzumab plus CRT arm)	Reddy <i>et al.</i> 2014 [67]
Lapatinib (EGFR/HER2 inhibitor)	Phase III plus BSC/MTX	Platinum-refractory recurrent/metastatic	Improved PFS	Machiels <i>et al.</i> 2011 [16]
Afatinib (irreversible ERBB-family blocker)	Phase II plus CRT	Locally advanced	Increased CRR and median PFS in p16-negative disease	Harrington <i>et al.</i> 2013 [72]
	Phase III trial vs. MTX	Recurrent/metastatic	Improved PFS	Machiels <i>et al.</i> 2015 [17]

*BSC* best supportive care, *CRR* complete response rate, *CRT* concurrent chemoradiotherapy, *CT* chemotherapy, *mAb* monoclonal antibody, *MTX* methotrexate, *OS* overall survival, *PFS* progression-free survival, *RT* radiotherapy

## 2.5 MicroRNA in HNSCC

Compelling evidence indicates that the human genome is regulated by microRNAs (miRNAs). miRNAs are short, noncoding RNAs that regulate transcription and translation of their target genes by binding to the highly evolutionarily conserved 3'-untranslated regions of mRNAs [95]. Altered expression of miRNAs correlates with human cancers [96], and several miRNAs are either up-regulated or down-regulated in HNSCC [97].

Up-regulated miRNAs, such as miR-21 (negatively correlated with PTEN) and miR-205 (which targets PTEN), promote cell proliferation by blocking cell cycle inhibitors, whereas down-regulated miRNAs, such as the let-7 family, negatively regulate KRAS [98]. miRNA are also involved in chemoresistance as revealed by levels of expression in resistant HNSCC cell lines [99].

### Outlook and clinical challenges

Further studies are warranted to investigate the use of miRNAs as diagnostic, prognostic, or therapeutic markers of HNSCC, but the high rate of abnormalities detected by genomic studies points to a previously unexpected role of these molecules in HNSCC. The tumor suppressor let-7c has been found to be altered in 40 % of HPV-negative and 17 % of HPV-positive HNSCCs [16], and specific miRNAs have also been associated with a propensity for metastasis and poor outcomes [100].

## 3 A new perspective in cancer treatment: targeting the tumor microenvironment

### 3.1 The tumor microenvironment in HNSCC

Numerous studies have demonstrated the essential role that the tumor microenvironment plays in the acquisition

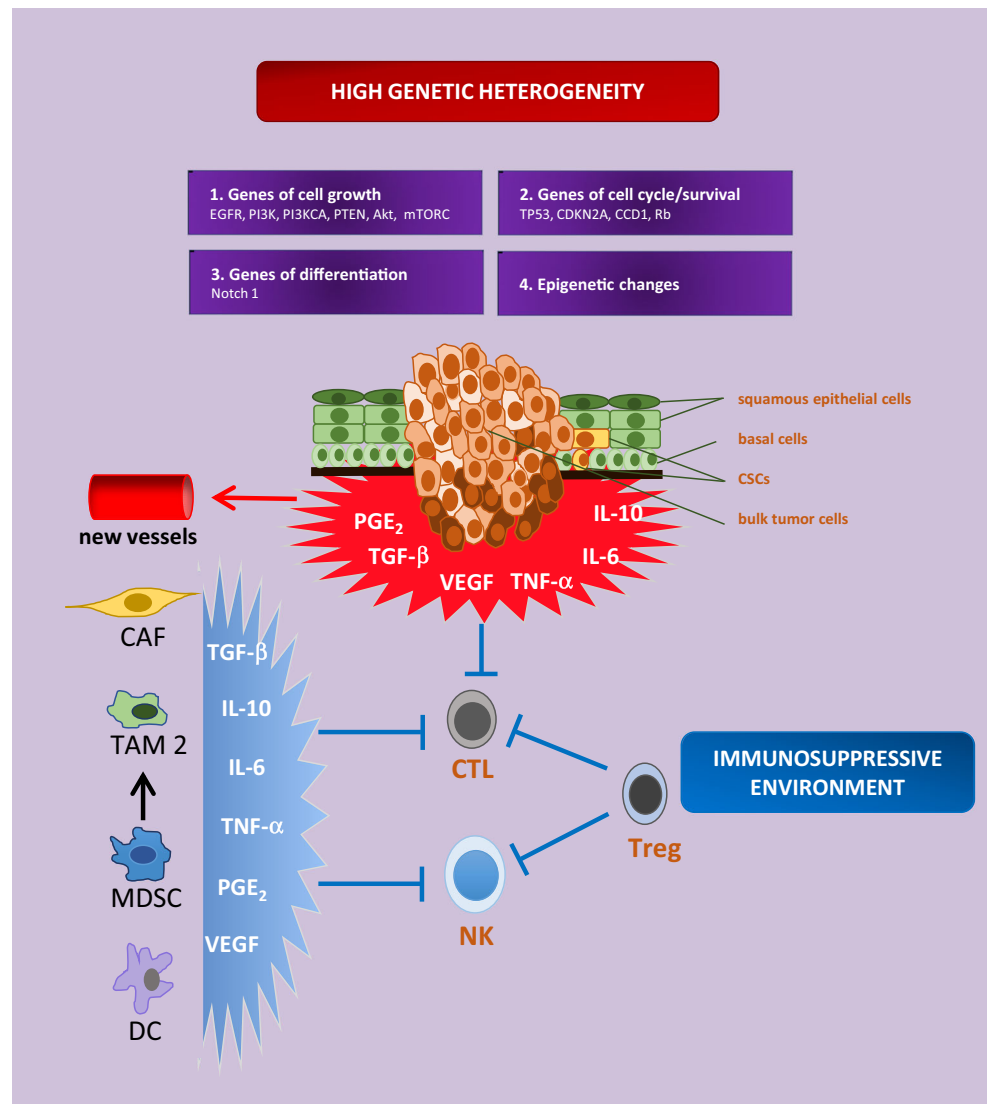
of hallmark capabilities [101]. The particular properties of the tumor microenvironment play a prevalent role in progression of HNSCC and represent potential targets for new therapeutic approaches, along with conventional or new molecular-driven therapies. The mucosa of the nasopharynx, oropharynx, and hypopharynx progressively changes from pseudostratified respiratory epithelium to a nonkeratinized stratified squamous layer. The oropharyngeal tract contains tonsillar lymphoid follicles in which the mucosa extends deep into crypts and alternates stratified squamous cells and reticulated sponge-like layers [102]. Reticulated patches associated with discontinuous basement membrane collect pathogens hiding in the crypts [103]. The mucosa is also enriched with basal cells localized near the basal lamina. Under normal conditions, these cells contribute to the slow turnover of the epithelium, but they may convert into cancer stem cells (CSCs) responsible for tumor initiation and progression (see [104, 105] for an exhaustive review of CSCs in HNSCC).

The tumor microenvironment of HNSCC, particularly the oropharyngeal tract, contains a predominance of nonepithelial cells, which provide support for growth factors, cytokines, and chemokines to promote invasiveness and chemoresistance (Fig. 1). These cells include lymphocytes, macrophages, dendritic cells, vascular cells, and stromal cells. Hereafter, we will discuss how some of these cells exert a suppressive role in the antitumor immune response (Fig. 2).

### 3.2 ROS, inflammation, and immunity

Tobacco use, alcohol consumption, and HPV infection trigger inflammatory and immune activation. Oxidative stress is a major effector in this process, as chemical carcinogens produce such a high level of reactive oxygen species (ROS) and reactive nitric species (RNS) that scavenging by antioxidants is always inefficient. Compelling experimental and clinical

**Fig. 1** Genetic heterogeneity and the immunosuppressive microenvironment characterize HNSCCs. Genes regulating cell cycle and survival, cell growth, and differentiation are frequently mutated or amplified in HNSCC. Epigenetic changes have also been identified as drivers of tumor progression. Cancerogenesis produces a bulk of heterogeneous cells, including cells with invasion and metastatic capacity. The tumor microenvironment is characterized by an abundance of cytokines and growth factors produced by tumors cells (escape mechanisms) and inflammatory, stromal, and antigen-presenting cells. Collectively, these cells provide an unfavorable milieu that inhibits the immunological response and promotes tumor growth and survival. *CAF* cancer-associated fibroblast, *CSC* cancer stem cell, *CTL* cytotoxic T lymphocyte, *DC* dendritic cell, *MDSC* myeloid-derived suppressor cell, *NK* natural killer, *TAM* tumor-associated macrophage, *Treg* regulatory T lymphocyte

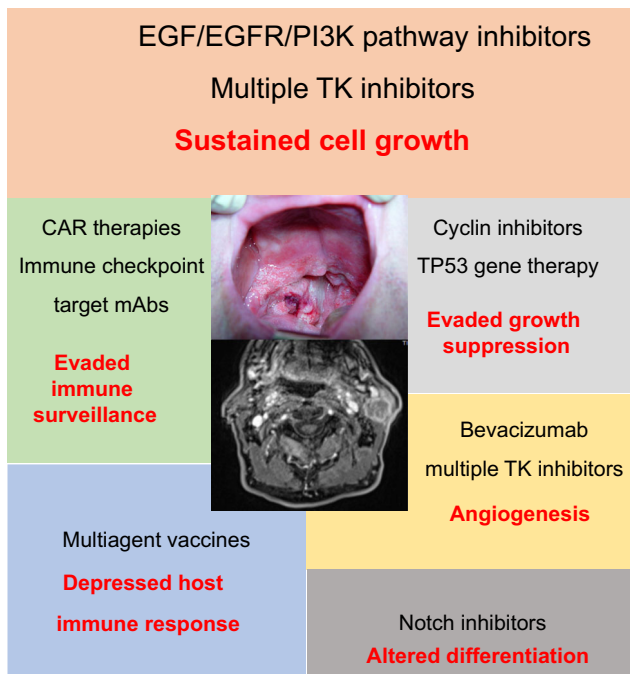


evidence indicates that ROS produce a broad range of effects, from genomic instability and changes in signaling pathways to activation of inflammation, tissue repair, controlling cell proliferation, and survival; affect cell motility and invasiveness; and activate inflammation, tissue repair, *de novo* angiogenesis [106], and differentiation of basal stem cells [107].

In HNSCC, the cross-talk between tumor and inflammatory cells is multifaceted, as demonstrated by the effect produced by tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) in tumor development. TAM subpopulation M1 promotes inflammation and exerts an antitumor function, whereas TAM subpopulation M2, the predominant variant in malignant proliferations, activates angiogenesis and tissue remodeling and sustains tumor progression [108]. Thus, concomitant to inflammation, monocyte-derived macrophages create a favorable environment for tumor growth by secreting EGF, PDGF, and

TGF-β [109]. Macrophages also synthesize the chemotactic factor macrophage inflammatory protein-3α, which drives HNSCC cell migration and invasion [110]. HNSCC patients with high levels of expression of M2 markers CD68 and CD163 present with significantly worse clinical outcomes [111], a finding that provides a rationale for targeting M2 depletion in HNSCC. M2 can also be generated by MDSCs. MDSCs are an intrinsic part of the myeloid lineage and are characterized by the capacity to suppress T cell responses in various ways. MDSCs also produce factors that support tumor growth and angiogenesis, stimulate M2 differentiation, and contribute to the production of an immunosuppressive milieu that favors tumor survival [112].

Head and neck tumor cells are actively eliminated by tumor antigen (TA)-dependent and TA-independent host immune responses. However, immune surveillance breaks down when tumor cells harbor escape mechanisms that allow them to



**Fig. 2** Principal hallmarks of HNSCC (*red text*) and corresponding therapeutic approaches (*black text*). Inhibitors of the EGF/EGFR/PI3K pathway are the most validated options in the clinical setting, but other promising therapies are under clinical testing or preclinical design. The image in the central area of the figure refers to locoregionally advanced oropharyngeal squamous cell carcinoma. *CARs* chimeric antigen receptors, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *mAbs* monoclonal antibodies, *PI3K* phosphoinositide 3-kinase, *TK* tyrosine kinase

avoid or inhibit the immune system. For example, tumors can co-opt certain immune checkpoint pathways used by the immune system to maintain self-tolerance, modulate the duration and amplitude of the immune responses, and avoid collateral tissue damage. Many of the immune checkpoints are initiated by ligand-receptor interactions, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1).

CTLA-4 receptor is expressed on T cells and attenuates T cell immune response through its ligands CD80 and CD84. PD-1 receptor is also expressed in activated T cells, APCs, and NK cells, and inhibits T cell activation through its ligands PD-L1 and PD-L2 [113]. The ligands PD-L1 and PD-L2 have broad expression ranging from T, B, and NK cells to some tumor cells, including those of HNSCC [34, 114]. PD-L1/2-Pd-1 interaction results in progressive exhaustion of the immune response. Ultimately, tumor immune evasion is mainly due to PD-1-positive T cells that infiltrate tumor bulks expressing high PD-L1 levels.

In HPV-positive HNSCC, immunosuppression is increased further by viral infection, which may explain why, paradoxically, these tumors commonly develop

within the immune tissue of tonsillar lymphoid follicles, an anatomic site that should favor immunologic antitumor response. Here, HPV blocks interferon-alpha, inhibits CTLs, activates suppressor T lymphocytes, and down-regulates expression of MHC complex I [115]. The immunosuppressive milieu produced by inflammatory cytokines maintains latent infection and favors tumorigenesis, which is initiated when the viral DNA integrates into the host genome and drives genomic instability. Once infected and transformed by HPV, tumor cells activate additional mechanisms to escape the immune system by preventing exposure of tumor antigens and promoting apoptosis of effector T lymphocytes and down-regulation of NK cells [116].

### Outlook and clinical challenges

In principle, many of the immune checkpoints can be blocked or modulated by monoclonal antibodies in order to release cytotoxic T cells from anergy and tolerance [113].

Ipilimumab, a monoclonal antibody against CTLA-4, was the first biological drug of this class to obtain FDA approval for its relevant clinical benefit in metastatic melanoma [117]. Since then, a number of immunotherapies have been also proposed for HNSCC, although the prognostic and predictive role of the expression of immune checkpoint biomarkers in HNSCC is still under debate [118–120].

Pembrolizumab, an anti-PD-1 monoclonal antibody, has been tested in the phase Ib Keynote 012 trial in recurrent/advanced HNSCC expressing PD-L1. In this study, response rate was nearly 20 % based on RECIST criteria, regardless of HPV status, and clearly correlated with PD-L1 expression level [121]. In an expansion cohort of the same study, tumor shrinkage was reported in 57 % of the patients with a response rate of nearly 25 % and acceptable toxicity [122]. Responses were durable, remarking the novelty of these results, compared to earlier experiences with cetuximab; longer follow-up is needed to assess survival.

Two phase III trials have been planned to compare pembrolizumab as single agent (or in combination with chemotherapy) with standard treatment in recurrent/metastatic HNSCC (ClinicalTrials.gov identifier: NCT02252042–NCT02358031).

Another phase III trial of nivolumab (a fully human antibody targeting the PD-1 receptor) in comparison to standard treatment in recurrent/metastatic HNSCC has been prematurely discontinued for the evidence of a superior survival for the nivolumab arm (ClinicalTrials.gov identifier: NCT02105636).

Promising results have been also reported in a multiarm dose expansion study employing the PD-L1 inhibitor durvalumab (MEDI4736). In 54 metastatic HNSCCs, not preselected for PD-L1 expression, the response rate was 14 %, reaching 50 % in the subset of PD-L1 expressing tumors [123]. A phase III open label study of durvalumab with or without tremelimumab (fully human monoclonal antibody targeting CTLA-4) *versus* standard of care in recurrent/metastatic HNSCC is ongoing (ClinicalTrials.gov identifier: NCT0255159).

To sum up, important and innovative features make the checkpoint inhibitors the current most promising therapeutic strategy in HNSCC for the relative high percentage of durable responses and the favorable toxicity profile.

Other immunotherapies have been designed to target immunological mechanisms involved in tumor progression. This is an area of intensive translational research with both promising successes and persistent disappointments. Besides the already illustrated line of research on



checkpoint inhibitors, four other major strategies have been translated from basic research to clinical trials (Table 2) [124–126, 128, 129, 131, 132, 138–144]: (1) conventional therapies that display immunomodulatory effects; (2) targeted therapies that, beyond the function of targeting oncoproteins, may play a role in tumor-mediated immunosuppression; (3) therapeutic vaccines used to stimulate an active immune response against a specific MHC-bound TA-derived peptide; and (4) autologous T cells engineered to produce special receptors (chimeric antigen receptors) that allow the T cells to recognize specific proteins on tumor cells.

### 3.3 Invasion and metastasis

HNSCCs are characterized by their propensity to spread *via* direct infiltration through lymphatic, haematogenous, or

perineural routes. Neck metastatic lymph nodes are quite common at presentation, with survival reduced nearly by half when they are present [136]. Metastatic dissemination involves several steps, most of which are coordinated by epithelial mesenchymal transition (EMT) and remodeling of the extracellular matrix. Cells undergoing EMT shift protein synthesis to overexpress cytoskeletal proteins that detach and invade the extracellular matrix through actin-rich protrusions and focal adhesions. In addition, the invasive borders of HNSCCs are enriched with cells that express matrix metalloproteinases (MMPs)—mainly MMP-9 and MMP-2—and actin-rich structures, called filopodia and invadopodia, that mediate ECM proteolysis [130]. The TGF- $\beta$  pathway is a key molecular player in EMT. TGF- $\beta$ , in cooperation with

**Table 2** Selected basic science and clinical evidence on immunotherapy for HNSCC

Therapeutic approach	Major evidence	Reference(s)
<b>Conventional therapies</b>		
Cisplatin	Collateral immunomodulatory effects: up-regulation of MHC class I, recruitment of T cells and TAMs, down-regulation of TREGs and MDSCs	de Biasi <i>et al.</i> 2014 [124]
Taxanes	Collateral activation of DCs, NK cells, CTLs; up-regulation of mannose-6-phosphate tumor cell receptors with increase of permeability to granzyme-B	Chang <i>et al.</i> 2013 [125]
5-Fluorouracil Radiotherapy	Collateral increase of IFN-gamma production by CD8 T cells Increase in type I IFNs with enhancement of both intratumor concentration of CXCR3 chemokine and activity of CD8 T cells	Tsuchikawa <i>et al.</i> 2012 [126] Lim <i>et al.</i> 2014 [127]
<b>Targeted therapies</b>		
Cetuximab	Collateral up-regulation of MHC II and costimulatory factors on DCs. Increase of immune responses: complement-dependent cytotoxicity, NK-mediated antibody-dependent cytotoxicity, macrophage-mediated antibody-dependent cellular phagocytosis.	Vannemann & Dranoff 2012 [128] Srivastava <i>et al.</i> 2013 [129] Kumai <i>et al.</i> 2014 [130]
Bevacizumab	Collateral enhancement of differentiation of DCs and blockade of MDSCs	Alfaro <i>et al.</i> 2009 [131]
Sunitinib	Blockade of secretion of IL-10 and TGF- $\beta$ and enhancement of production of IFN-gamma by tumor T cells	Alfaro <i>et al.</i> 2009 [131] Ozao-Choi <i>et al.</i> 2009 [132]
<b>Cancer vaccines</b>		
Multiagent vaccines	Specifically target TAs: Ly6k (lymphocyte antigen 6 complex locus), CDCA1, IMP3 (insulin-like growth factor II m-RNA-binding protein). Phase II trial: improvement in OS in HLA*24:02+ advanced HNSCC patients	Yoshitake <i>et al.</i> 2015 [133]
DC-based wild-type p53 peptide vaccine	Induction of antitumor response by T cells. Phase I trial: treatment safe, with promising clinical outcome	Schuler <i>et al.</i> 2014 [134]
<b>CAR therapies</b>		
Targeted CAR therapy	LMP1/CAR (latent membrane protein) CSPG-4 CAR (chondroitin sulfate proteoglycan-4). Promising results in preclinical evaluations.	Tang <i>et al.</i> 2014 [135] Geldres <i>et al.</i> 2014 [136]
<b>Immune checkpoint target therapies</b>		
Monoclonal antibodies	Anti-PD1–anti-PD-L1 Treatment safe. Remarkable results in preliminary phase I studies. Up to date, no definitive results in terms of clinical outcome in HNSCC. Pembrolizumab and nivolumab under evaluation in a phase III trial.	Swanson <i>et al.</i> 2015 [137] Seiwert <i>et al.</i> 2014 [138] Seiwert <i>et al.</i> 2015 [139] Segal <i>et al.</i> 2014 [140]

CAR chimeric antigen receptor, CTLs cytotoxic T lymphocytes, DCs dendritic cells, IFN interferon, IL interleukin, MDSCs myeloid-derived suppressor cells, MHC major histocompatibility complex, NK natural killer, OS overall survival, TA tumor antigen, TAMs tumor-associated macrophages, TGF tumor growth factor, TREGs regulatory T cells



its cognate receptors and transducers (SMAD2, SMAD3, and SMAD4), activates genes of cell motility and down-regulates epithelial genes [127]. TGF- $\beta$  is secreted by tumor cells and by a number of cells of the tumor microenvironment, including cancer-associated fibroblasts and TAMs.

Another pathway associated with proliferation and migration of tumor cells involves Src, a cytoplasmic tyrosine kinase, activated by a number of growth factors, including EGFR, FGFR, and VEGFR [134].

#### Outlook and clinical challenges

Clinical evidence supporting the targeting of metastatic dissemination in HNSCC has been elusive. Studies using sarcatinib (AZD0530), a small molecule inhibitor of Src, in combination with either the phospholipase C inhibitor U73122 or the EGFR inhibitor gefitinib found reduced cell invasion *in vitro* [135, 137], but clinical trials have failed to demonstrate any significant benefit [133]. Broad-spectrum MMP inhibitors have also been used, with very limited success in most cancers [145].

### 3.4 Angiogenesis

Angiogenesis is a well-known factor that is necessary for nourishing tumor cells and CSC niches and for promoting metastatic progression [146]. Angiogenesis is supported by hypoxic response or inflammation [147] as well as a variety of factors in the tumor microenvironment, such as VEGFR and NF- $\kappa$ B. VEGF enhances endothelial growth, migration, and differentiation. Its overexpression has been detected in up to 40 % of cases of HNSCC and is associated with poor prognosis [148].

#### Outlook and clinical challenges

Targeted therapies to inhibit angiogenesis include monoclonal antibodies anti-VEGF and multikinases inhibitors, such as sunitinib and the aforementioned sorafenib. Bevacizumab, an anti-VEGFR monoclonal antibody, has been tested in phase II trials in combination with other molecular targeted therapies or chemotherapy and has shown interesting levels of activity [149–151]. Unfortunately, a significant number of bleeding events (some of which were fatal) have been reported, suggesting that evaluation on the dose to be used and patients' selection has to be reconsidered. Results are also anticipated from trials investigating multiple tyrosine kinase inhibitors. At present, phase II studies have reported stable disease as better response, with an encouraging PFS and toxicity profile for sorafenib [75, 152]. Two controlled trials with chemotherapy associated with bevacizumab (ClinicalTrials.gov identifier: NCT00588770) or sorafenib (ClinicalTrials.gov identifier: NCT02035527) are ongoing.

### 4 Conclusions

HNSCC is an extremely heterogeneous disease with distinct patterns of presentation and biological behavior. Patients with HNSCC are frequently treated with aggressive treatment

strategies that may strongly affect quality of life and elicit unpredictable results. The success of EGFR-targeting therapies combined with radiation or chemotherapy covers a limited number of cases. For this reason, it is essential both to explore new multi-strategy approaches, by the use of combined genetic and cellular targeted new therapies, and to investigate potential predictive biomarkers for treatment response. To date, only HPV is likely to be a prognostic indicator and a predictive marker of response to treatment. Recent whole-exome sequencing studies have provided a comprehensive view of the genetic alterations and the complexity of gene mutations underlying this malignancy. Although few driver genes are currently targetable, and although the predominance of tumor suppressor gene alterations presents a challenge for the treatment of HNSCC, these investigations, as well as new insights into the tumor microenvironment, have provided a deeper and comprehensive understanding of HNSCC biology establishing a basis for potential molecular recognition-based customized therapeutic approaches. In particular, the immune checkpoint inhibitors represent the most promising strategy for HNSCC in the next future.

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