Gene alterations in head and neck carcinomas and their role in promoting malignant behavior (Review)

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Abstract. Head and neck squamous cell carcinoma (HNSCC) survival remains poor despite continuing efforts toward prevention, early detection, and improved treatment modalities. In part, this is thought to be due to a relative lack of molecular targeted therapeutic strategies beyond general mitosis inhibition, which sets a limit to what modern head and neck surgery can accomplish for advanced disease. The past 30 years have produced a large quantity of data, leading to a better understanding of HNSCC carcinogenesis and novel therapeutic agents, such as epidermal growth factor receptor blockers. This article reviews literature on the current understanding of molecular HNSCC carcinogenesis, and highlights the most promising therapeutic approaches.

Contents

1. Introduction

Squamous cell carcinoma of the head and neck is the sixth most common cancer worldwide. While potentially curable at an early stage, more than 40% of patients present with locally advanced or metastatic disease at the time of diagnosis, with an overall 5-year survival rate between 40-63% (1). HNSCC mortality remains largely unimproved despite ongoing advancements in tumor surgery, as well as radio- and chemotherapy (2). A part of this problem is thought to be the relative lack of targeted radio- and chemotherapy regimens selected based upon tumor biological properties. The remarkable success of this type of targeted therapy has been demonstrated in BCR-ABL fusion protein blockade in chronic myeloid leukemia (3). It is understood that tumor prognosis heavily depends upon cancer cell biology. For example, HNSCC arising from human papilloma virus infections are associated with a relative survival advantage (4). Considerable research efforts have been dedicated to study the molecular biology of HNSCC. Over the last three decades, several cancer related transcripts have been suggested as factors of etiological importance. Some were recognized as biomarkers to guide treatment decisions, while others became targets for the development of 'intelligent drugs' (5). This article reviews molecular findings of HNSCC carcinogenesis and organizes them based upon the hallmarks of malignant tumors by Hanahan and Weinberg (6), as well as other important pathophysiological principles. Our aim is to discuss these findings in light of their significance for molecular disease mechanisms which are important for the development of novel therapeutic agents.

2. Oncogenes and tumor suppressor genes

Genetic anterations of oncogenes and tumor suppressor genes can affect their expression or may produce an altered gene product resulting in malignant transformation of the cell (reviewed in refs. 7-9). Altered expression of oncogenes has been considered as a prognostic factor in a variety of tumors. Particularly, upregulation of the *myc* genes has been reported to be prognostic indicators in different tumor entities. Kiaris *et al* showed that genetic instability of a repetitive element which is located within intron 1 of the H-*ras* gene is associated with the nodal status of patients with HNSCC (10,11). Spandidos *et al* found significant activation of multiple oncogenes (H-*ras*, K-*ras*, c-*myc*, *fes*, *abl*, *sis*) in premalignant and malignant head and neck solid tumors (12), and Field *et al*

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proposed that the enhanced expression of c-*myc* which was correlated with advanced stages of the disease, may be an effective prognostic indicator in head and neck cancer (13,14).

In different neoplasias genetic alterations have mostly been studied to determine allelic imbalance or loss of heterozygosity to identify chromosome regions that may contain tumor suppressor genes. Field *et al* identified two regions in HNSCC that are most likely to be important in the development of head and neck carcinoma at 3p24-p25 and 3p13 and may indicate sites of novel tumor suppressor genes in this tumor entity (15). In a comprehensive study Field *et al* demonstrated a complex set of genetic alterations, in HNSCC where the highest loss of heterozygosity was found on the chromosome arms 3p, 9p, 17p and 18q (16). Similar observations were made by Nawroz *et al* (17) and Ah-See *et al* (18) indicating the importance of the analysis of accumulation of genetic aberration in head and neck tumors.

In a wide range of human cancer, an important factor controlling cellular growth is p53. The mechanisms responsible for loss of p53 in HNSCC can be divided into mutation of the p53 gene (about 50%), inactivation by p53 modulators, or targeted degradation (as in the case of Human Papilloma Virus early E6 protein (19). Its loss of function is a characteristic early change frequently observed as a precursor to HNSCC (35), however, the p53 gene also have a role in the late stages of this disease (20). Field *et al* found, that mutations in the p53 gene at codon 249 are rare in HNSCC in contrast to hepatocellular carcinomas and their observations indicate that the hot spot mutations have occurred in the 238-248 region (20,21). Interestingly, p53 overexpression and mutations correlate with alcohol and tobacco abuse of patients with HNSCC (22,23).

P53 can suppress carcinogenesis by several mechanisms. In response to a myriad of stress signals, p53 pathways can lead to two principal outcomes: repair of the acquired damage via initiation of a cell cycle arrest (G1/S) and resultant DNA repair, or self-destruction via apoptosis (24). For those types of cancer expressing wild-type p53 rendered non-functional by modulation or accelerated degradation, therapeutic options exist in utilizing the inherently functional p53 to a higher degree. This can be achieved by inhibiting the interaction of p53 with its central inhibitor, human double minute 2 (25). Another important part of growth inhibition is mediated via cell-cell contacts. In HNSSC such effects can be induced by adhesive glycoproteins facilitating cellular adhesion, attachment to extracellular matrix (ECM) and contact inhibition (26-28).

3. Self-stimulation via growth signals

Cellular growth factors are indispensible for mitosis. However, their signal cascade is also a chief pathway of malignant signal transduction. Alteration of extracellular growth signals, their cellular transmission, or intracellular circuits that translate those signals into action all can lead to malignant transformation (6). Stimulation of epidermal growth factor receptor (EGFR) and its associated pathways has long been suspected to undergo such alterations in the development of HNSCC (29). In 1997, Issing found that patients with simultaneous expression of transforming growth factor α (TGF-α) and its cell surface receptor EGFR had a poor prognosis. He hypothesized an autocrine loop, in which HNSCC cells synthesize their own growth factor (30). Grandis *et al* demonstrated a decrease in TGF-α production via antisense oligonucleotides which reduced cellular proliferation rate (31). EGFR itself has been described as inducible by carcinogenic growth factors leading to alteration of extracellular, as well as transcellular signal transduction (32). Subsequently, EGFR expression was investigated as a prognostic marker, and found to be associated with poor outcomes (33). EGFR has progressively become an important target of direct antineoplastic therapy, especially via the chimeric monoclonal EGFR antibody Cetuximab (34). In 2006, Bonner *et al* reported that the addition of Cetuximab to radiotherapy significantly prolonged locoregional control and decreased mortality compared to radiotherapy alone (35). Vermoken *et al* reported a phase III trial of Cetuximab as first-line treatment together with platinum and fluorouracil to treat patients with recurrent or metastatic HNSCC (36). As a result of these trials, Cetuximab has become an established component of HNSCC treatment.

Adverse effects of Cetuximab in HNSCC are still under investigation. From the colon cancer literature, known side effects of Cetuximab monotherapy are allergic reactions, acneiform rash, asthenia/malaise, fever, pulmonary and gastrointestinal symptoms (37). In addition, Cetuximab is described as causing hypomagnesemia due to inhibition of renal tubular reabsorption. However, this hypomagnesemia is posited to have antineoplastic effects (38). Giro *et al* recently observed a higher rate of radiation dermatitis in patients with HNSCC who were treated with Cetuximab and radiation therapy (39). It is conceivable that EGFR blockade impairs the regeneration of other tissues after surgery and radiochemotherapy. Continuous efforts to improve the side effect profile have been made, including research on other monoclonal antibodies against EGFR. Notably, the human monoclonal EGFR antibody Panitumumab has been studied and FDA approved for colorectal cancer (40). The principal advantage of Panitumumab is that it is a fully human monoclonal antibody, eliminating antibody formation against itself (41). At the same time, Cetuximab may elicit immune responses specifically for the mouse portion of the molecule leading to an immune reaction against the antibody and targeted tumoral cells (42). Panitumumab is currently being investigated in HNSCC patients as well.

Over time, compared to the high expectations that accompanied the introduction of anti EGFR therapy, results have been modest. Only 15% of the patients treated showed a clinical response, and these drugs contributed only to a relatively small overall survival benefit (42). One of the reasons for these results is a still incomplete understanding of the molecular mechanisms promoting HNSCC. The chief mechanism of action for both Cetuximab and Panitumumab is inactivation of EGFR activated signaling. That has important implications, as mutations in intracellular signaling molecules downstream of EGFR such as in the g-protein K-ras can infer resistance to EGFR receptor blocker treatment (43).

EGFR-independent mechanisms, including soluble insulin growth factor 1 (IGF-1) and the intracellular nuclear factor-κB

(NF-κB), also contribute to the activation of key intracellular signaling routes in HNSCC growth (44,45). Slominary *et al* proposed interrupting intracellular signaling of both the EGFR and IGF-1 systems, through targeted inhibition of the tyrosine kinases of both IGF-1R (NVP-AEW541) and EGFR (AG1478) (46). However, results so far exist only on a preclinical level.

4. Tissue invasion and metastasis

Local invasion and distant metastasis, rather than mere cell proliferation, are the key determinants of both morbidity and mortality for a vast majority of tumors. These two processes cause 90% of cancer related death (48). Mechanisms include dislodgement of HNSCC cells via loss of cell-to-cell contacts, secretion of enzymes facilitating dissolution of the basal lamina and other ECM structures, and tumor cell migration. In HNSCC, cellular dislodgement has been reported to be facilitated via loss of adhesion proteins, such as E-cadherin (49), claudin-7, connexin 31.1 (50), or FN (47). However, it also can occur secondary to expression of glycoproteins interfering with cellular adhesion, such as dysadherin (51).

ECM and basal lamina dissolution are in major part attributed to matrix metalloproteinases (MMP) being capable of cleaving ECM and basilar membrane macromolecules. In HNSCC high MMP expression was found to be associated with regional lymph node and distant metastases (52), and a higher rate of recurrence (53). Highly expressed MMP was also found in a variety of cancers, implicating their importance in tumor invasiveness and metastasis formation (54).

To this date, there is no clinical trial on MMP inhibitors in HNSCC published to the best of our knowledge. By analyzing the reasons for the relative lack of successful clinical trials despite promising pre-clinical data, Rosenthal and Matrisian suggested that mouse models used in preclinical studies with xenotransplanted tumor tissue may be inadequate, as these mice are immunodeficient. Additionally, their MMP genotype and phenotype is quite different from humans. MMP inhibitors may also fail as monotherapy, as they have little cytotoxic effects. Dosing was limited by musculoskeletal side effects, and thus it remains unclear whether other modalities of applying the medication could be beneficial (55).

ECM composition is regulated by a variety of factors other than MMP. The lysyl oxidase (LOX) enzymes are one of the prominent factors that have gained interest. LOX are secreted copper-dependent amine oxidases that catalyze the oxidation of peptidyl lysine to δ-aminoadipic ß-semialdehyde. This is an intermediate step in the formation of covalent cross-linkages between elastin and collagen, which is essential in the development and maintenance of the ECM (56). Consequentially, LOX were hypothesized to function as tumor suppressors by limiting cellular invasion. However, over time, the analysis of LOX family genes in cancer cell lines was found to be much more heterogeneous. Comprehensive investigations on gastric (57), colon (58), prostate (59), and breast cancer (60) indicated both decreased, as well as increased LOX gene expression. Interestingly, both LOX and lysyl-oxidase-like 4 (LOXL4) mRNA were found up-regulated in HNSCC (61,62). We found LOXL4 expression increased within the HNSCC and not the surrounding stroma cells (62,63). This suggests a role in promoting tumor invasion and metastatis formation.

Further research on the LOX enzyme indicated other functional regions apart from its highly conserved copper and lysyl-tyrosyl-chinon (LTQ) binding domains necessary for the collagen/elastin linkage. These included regions promoting chemotactic responses, proliferation, and shifts between the normal and malignant phenotypes (64). This led to the hypothesis that intracellular and extracellular LOX may have entirely different functions. Erler *et al* showed a link between tissue hypoxia, the expression of LOX, and metastasis formation (65). In HNSCC, this correlation was confirmed in case of LOX expression as a poor prognostic marker (63,66).

These findings have led to preclinical studies on therapeutic agents that might be utilized in cancer treatment. Notably, Bondareva *et al* investigated ß-aminopropionitrile in mice that received breast cancer cells. They found that this agent could reduce the rate of new metastasis formation. However, the growth of existing metastases was not affected (67). In a feasibility study, we transfected cloned LOXL4-mRNA into immature dendritic cells and showed that cellular expression of LOXL4 could provide an effective target for cell mediated immunotherapy (68). Another important aspect of metastasis formation is the phenomenon of directional movement, which is well characterized for many cells, particularly leukocytes. In 2004, Wang *et al* reported upregulation of chemokine receptor 7 on head and neck cancer cells as a novel mechanism of metastasis formation that could help 'home in' towards lymph nodes, and thus aid metastatic spread (69).

5. Limitless self-replicative potential

While the independence from external regulatory signals is a precondition for a cancer cell to develop replicative autonomy from its environment, this alone does not suffice to overcome other natural limitations of redundant mitosis, such as a terminal phase called crisis at the end of numerous replications (6). Wright *et al* observed that one in 10^7 cells can spontaneously acquire the ability to overcome this natural limitation and form an 'immortalized cell' that has the ability to replicate itself endlessly (70).

In HNSCC, Mao *et al* showed that activation of telomerase, the enzyme responsible for elongating telomeres and thus enabling the cell to bypass the Hayflick limit, is frequent in HNSCC and may occur early in the neoplastic process (71). Thurnher *et al* suggested that elevated telomerase activity in HNSCC correlated with lymph node metastasis (72). While telomerase is a tempting target for cancer treatment, a number of anti-neoplastic agents and plant products were found to interfere with its function in hindsight (73,74). There is evidence that telomerase activity is regulated by caspase activity, and that factors promoting apoptosis also reduce telomerase activity (75). New therapeutic attempts to decrease telomerase activity in HNSCC cells have been made via ceramide application (76).

6. Evading apoptosis

Tumor progression not only depends on cell replication and growth as delineated above, but also on cell survival,

Table I. Targeted therapeutic agents in HNSCC.

Therapeutic target	Compound	State of investigation in HNSCC[a]
EGFR	Cetuximab	Established in HNSCC
EGFR	Panitumumab	Established in colon cancer, six phase II, and three phase III trials in HNSCC
IGF-1R tyrosine kinase	NVP-AEW541	Preclinical
EGFR tyrosine kinase	AG1478	Preclinical
NF-κB	Diferuloylmethane	Preclinical in HNSCC, several phase III trials in other cancers
NF-κB	Curcumin	Preclinical in HNSCC, several phase III trials in other cancers
MDM2-p53 interaction inhibitors	Nutlins	Preclinical
MDM2-p53 interaction inhibitors	MI-219	Preclinical
MMP-2	Marinastat	Preclinical in HNSCC, three phase 3 trials in other cancers
MMP-2	Prinomastat	Preclinical in HNSCC, two phase III, one phase II trial
LOX	ß-aminopropionitrile	Preclinical
Apoptosis induction, telomerase	Ceramide	Preclinical in HNSCC, two phase II trials in other cancers
VEGF	Bevacizumab	Established in glioblastoma multiforme, in HNSCC: one phase III trial, 16 phase II trials and three phase I trials
VEGF	Aflibercept	One phase II trial, one phase III trial in other cancers
VEGF receptor tyrosine kinase	Sorafenib	Established in renal and liver cancer, in HSCC: two phase I, one phase II trial

[a]State of investigation as reported on clinicaltrials.gov accessed October 2009, including active as well as completed or suspended trials.

maintaining a replication velocity greater than the cell rate of cell loss (6). In HNSCC, Yang *et al* showed that activation of the Wnt/ß-catenin signaling pathway promoted cell survival, by avoiding apoptosis, as well as invasive growth of HNSCC cells (77). Sniezek *et al* demonstrated δNp63α upregulated in tumors and underexpressed in the pro-apoptotic condition of lichen planus. This suggests an anti-differentiation and anti-apoptotic role in the mucosal epithelium of the head and neck, possibly playing a pivotal role in the formation of HNSCC (78). We and Chiang *et al* reported that the proto-oncogene Pim-1, which functions to prevent premature onset of apoptosis, is upregulated in oral squamous cell cancer. This suggests an involvement into HNSCC carcinogenesis (79, 80). Pim-1 acts as a MYC cofactor, phosphorylating the chromatin at MYC-target loci. This has been suggested to contribute to MYC-dependent transcriptional activation (81).

Another gene involved in evading apoptosis in HNSCC is the lysosomal enzyme galactocerebrosidase (GALC), responsible for the hydrolysis of numerous monohexidose glycosphingolipids. Glycosphingolipids are known to be increased on the surface of cancer cells and play an important role in inhibition of cellular adhesion and apoptosis (82). An increase in the concentration of glycosphingolipids on the cellular membrane of cancer cells leads to significant changes of the antigenic properties. These include the formation of tumor associated carbohydrate antigens, loss of adhesion, and increased cellular motility (82). Galactocerebroside inhibits the induction of apoptosis, whereas the product of its hydrolysis via GALC, ceramide, promotes it (83). We could show that GALC is repressed in HNSCC (84), which fits into a carcinogenesis model in which the metabolism of HNSCC cells is modified to inhibit apoptosis and promote loss of cell adhesion (85).

The role of ceramide as an enhancer of apoptosis has already found application in cancer research (86). Reynolds *et al* showed that ceramide has cytotoxic effects. Exposure of cells to radiation or chemotherapy is associated with increased ceramide levels due to enhanced *de novo* synthesis, catabolism of sphingomyelin, or both (87). Several approaches have been undertaken to increase the level of ceramide

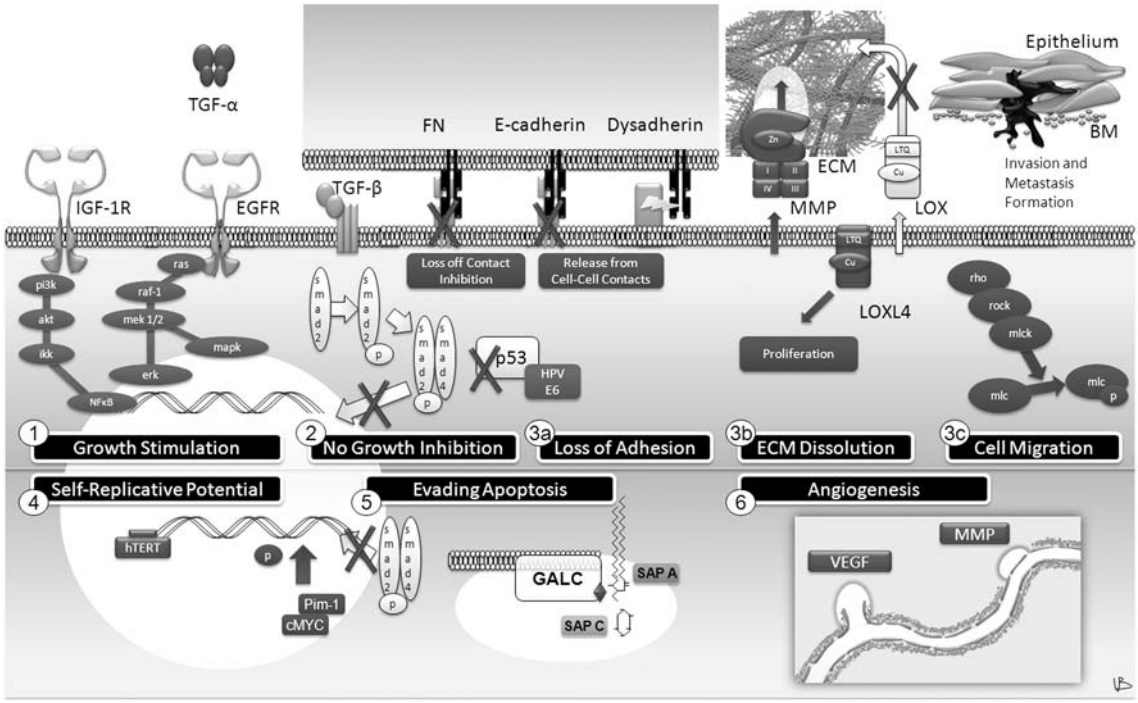

Figure 1. Molecular mechanisms promoting malignant behavior in HNSCC: growth stimulation (1) mediated via the epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF-1R) system. Resistance to growth inhibition (2) signals mediated by transforming growth factor (TGF) ß, which also affects lysyl-oxidase (LOX) expression and apoptosis. Tumor invasion and metastasis formation (3) is mediated by loss of cell-cell and cell-ECM (extracellular matrix) contacts (3a), enzymatic disassembly of barrier ECM structures such as the basement membrane (BM), in which matrix metallo-proteinases (MMP) play an important role (3b), and cellular migration mediated by contractile filaments (3c). Immortalization of the cell (4) and evasion of apoptosis (5) are closely linked. Galactocerebrosidase (GALC) facilitates the separation of galactose and ceramide, and thus may help in promoting apoptosis. Pim1 is an activated oncogene involved in the suppression of MYC-induced apoptosis by interacting with MYC and mediating the phosphorylation of histone H3. Angiogenesis (6) in two different steps, disassembly of the existing vascular basement membrane [by MMP, see (3)], and, migration and growth along the provisional vascular matrix to form a new blood vessel branch, stimulated by vascular endothelial growth factor (VEGF) and others. Abbreviations: pi3k, phosphoinositide 3-kinase; akt, proteinkinase B; IKK, inhibitor κB kinase; NF-κB, nuclear factor-κB; mapk, mitogen-activated protein kinase; FN, fibronectin; hTERT, human telomerase reverse transcriptase, HPV E6, Human Papilloma Virus early protein 6; SAP, saposin.

within tumor cells, including direct ceramide application in *in vitro* and animal studies, stimulation of ceramide *de novo* synthesis, sphingomyelinase activity stimulation, and inhibition of ceramide utilization (87,88). In HNSCC, Gu *et al* showed promising anticancer effects of vitamin E succinate to inhibit HNSCC growth and viability via ceramide-mediated apoptosis. This was found in both cancer cells and in a xenograft cancer mouse model (89). Other groups have attempted to find ways to deliver ceramide into HNSCC cells successfully (90).

7. Angiogenesis

Tumor growth depends on a sufficient supply of oxygen and nutrition, and therefore requires an ability to induce angiogenesis (91). The current conceptual framework of molecular angiogenesis divides the process into two principal phases (92): First, structural changes of the vascular basement membrane via tumor secreted MMP and growth factors leads to a remodeled vascular basement membrane (provisional matrix). In a second phase, the actual extension of the vascular bed, there is formation of a new vascular basement membrane in response to secreted vascular endothelial growth factor (VEGF), basic fibroblast growth factor and platelet-derived growth factor.

In HNSCC, secreted VEGF has been shown to induce endothelial proliferation, which, in combination with anti-proliferative TGF-ß, helps to form the anatomical space needed for angiogenesis. Its interaction with prostaglandin E2, inducing cellular locomotion, also could contribute to angiogenesis (93). EGFR directed therapy is, apart from interrupting autocrine growth signal stimulation, thought to inhibit VEGF release and thus also interferes with tumor angiogenesis (94).

There is also an extensive amount of research effort targeted directly against angiogenesis. These therapies can be divided into two major groups: The first group consists of direct VEGF antibodies (Bevacizumab) and soluble VEGF receptors (Aflibercept) which deplete VEGF and make it unavailable to promote angiogenesis. Overall, this approach has shown a benefit (95), although there is concern that diverse gene alterations affecting post-receptor signaling could blunt the response in some patients, analogous to the K-ras mutation in EGFR receptor blockers. Recently, Newman *et al* showed that in a HNSCC xenograft model, the response to Bevacizumab was dependent upon concomitant expression of CD147 (96). The second group of anti-angiogenic drugs targets the intracellular portion of the VEGF receptor and prevents its phosporylation (97).

8. Escaping the immune response

Immunotherapy for HNSCC is a very attractive treatment option of growing importance. HNSCC arise in a highly

immune reactive environment. Their survival therefore necessitates several mechanisms to evade the immune system. These rely on avoidance of detection (e.g. by down-regulating HLA expression), induced apoptosis of surrounding T-cells, and local suppressive immune modulation (98). In addition to direct antibodies against EGFR and VEGF mentioned above, immune modulators enhancing the host immune response such as interferon α and interleukin-2 could bear significant weight in treatment regimens. Another approach is the development of antibodies targeting antigens either overexpressed, or exclusively present in cancer cells. Several immune modulating agents and experimental antibodies which are summarized in Table I, are under investigation.

9. Conclusion

With regard to the recent cytogenetic studies multiple signaling pathways are activated in HNSCC (summarized in Fig. 1). Of the enormous amount of research done on identifying molecular pathogenesis and eventually developing therapeutic agents in HNSCC, EGFR antibodies are so far the most advanced in application. However, results using these agents have fallen below expectations. With the emerging recognition of the importance of post-EGFR signaling in predicting susceptibility to EGFR antibody treatment has illuminated some of the possible reasons for these unmet expectations. Much work remains in analyzing and understanding the molecular pathways promoting malignant behavior in HNSCC. On a positive note, there are a variety of other promising targets in multiple disease mechanisms, and knowledge about them is expanding at a rapid pace. From an overall perspective, halting invasion and metastasis formation alone could be sufficient to give rise to substantial clinical improvement, as these pathways are primarily responsible for a large proportion of morbidity and mortality in HNSCC.

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