Cetuximab chemotherapy resistance: Insight into the homeostatic evolution of head and neck cancer (Review)

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Abstract. The complex evolution of genetic alterations in cancer that occurs *in vivo* is a selective process involving numerous factors and mechanisms. Chemotherapeutic agents that prevent the growth and spread of cancer cells induce selective pressure, leading to rapid artificial selection of resistant subclones. This rapid evolution is possible because antineoplastic drugs promote alterations in tumor-cell metabolism, thus creating a bottleneck event. The few resistant cells that survive in this new environment obtain differential reproductive success that enables them to pass down the newly selected resistant gene pool. The present review aims to summarize key findings of tumor evolution, epithelial-mesenchymal transition and resistance to cetuximab therapy in head and neck squamous cell carcinoma.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of human cancer with ~750,000 cases worldwide, which may increase to 1 million by 2030 (1). HNSCC affects subsites of the upper aerodigestive tract, including the oral cavity, larynx and pharynx, and the main associated etiological factors are the use of alcohol and tobacco, and high-risk human papillomavirus (HPV) infection (2). The tumor suppressor *TP53* is one of the most frequently mutated genes in HNSCC. Results of The Cancer Genome Atlas (TCGA) HNSC project (https://portal.gdc.cancer.gov/) (3) revealed *TP53* somatic mutations in >90% (357/392) of the cases examined.

HNSCC exhibits heterogeneous tumor phenotypes, as a result of the reprogramming of the molecular machinery associated with carcinogenesis. According to Leemans *et al* (4), at least two genetic subclasses are identified in HNSCC regarding HPV infection status: i) Tumors with transcriptionally active HPV that are mostly located in the oropharynx and generally exhibit wild-type *TP53* alleles and a favorable prognosis; and ii) HPV-negative tumors that often present with high chromosomal instability, mutated *TP53* and unfavorable prognosis. Low numbers of numerical genetic changes and wild-type *TP53* are also observed in a group of HPV-negative lesions. Puram *et al* (5) demonstrated that the HPV-positive group may likewise exhibit high levels of chromosomal instability, as well as diversity in HPV expression, and in cell cycle and senescence states within and between tumors.

HPV-negative and -positive cases differ with respect to other characteristics. For instance, alterations in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) are frequently observed in HPV-negative tumors, whereas loss of TNF receptor associated factor 3 (*TRAF3*) and amplification of E2F transcription factor 1 (*E2F1*) occur frequently in HPV-positive tumors (6). *CDKN2A* (2) and *E2F1* (7) have regulatory roles in cell cycle progression, whereas *TRAF3* is involved in the activation of immune and inflammatory responses (8,9). Comparing the two groups, the mutational spectrum analyzed by Seiwert *et al* (10) also differs: Mutations in *TP53*, *CDKN2A*, cullin 3, discoidin domain receptor tyrosine kinase 2, F-box and WD repeat

domain containing 7 (*FBXW7*), lysine methyltransferase 2D/2C (*MLL2*/3), nuclear receptor binding SET domain protein 1, notch receptor 1 (*NOTCH1*), and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*); loss of 3p; and amplification of 11q13 and 7p11 potentially targeting cyclin D1, epidermal growth factor receptor (*EGFR*) and *CDKN2A*, respectively, in HPV-negative cases, and mutations in DEAD-box helicase 3 X-linked, *FBXW7*, fibroblast growth factor receptor 2/3, KRAS proto-oncogene GTPase, *NOTCH1* and *PIK3CA* in HPV-positive cases.

The molecular pathogenesis of HPV-positive HNSCC is driven through two viral oncoproteins, E6 and E7. E6 and E7 are overexpressed preceding or just after virus integration (11). E7 triggers the degradation of the tumor suppressor protein retinoblastoma, releasing E2F and consequently activating genes that promote the G1-S transition of the cell cycle. In turn, E6 induces the degradation of the tumor suppressor p53, a protein that promotes cell cycle arrest, apoptosis and DNA repair. These events may cause mutations, interchromosomal rearrangements and synthesis of abnormal transcripts, explaining the increased cell proliferation and genomic instability that accelerates the neoplastic process (12).

In addition to the main role of HPV in the development of oropharyngeal carcinomas, other viruses, as well as bacteria and fungi, are related to HNSCC etiology, albeit several of them with a less direct line of evidence. These examples include Epstein-Barr virus (EBV) and torque teno virus, which are associated with nasopharyngeal and laryngeal carcinomas, respectively. Bacteria and fungi of the oral microbiome appear to be associated with mouth neoplasms through the production of carcinogenic metabolites and the conversion of ethanol into mutagenic and carcinogenic acetaldehyde or promotion of hypermethylation, proinflammatory events and hypoxic or acidic environments (13). Such mechanisms evidence the link between poor dentition or oral hygiene and higher risks of HNSCC (14).

The initiating events of head and neck tumorigenesis in HPV-negative lesions are triggered by exposure to alcohol- and tobacco-derived carcinogens, differing from HPV-positive cases. Acetaldehyde and tobacco-derived carcinogens (polycyclic aromatic hydrocarbons and nitrosamines), as well as the resultant inflammation in exposed tissues, are mainly responsible for these events. Although the carcinogens are metabolized and excreted, their metabolites form DNA adducts, which, if not repaired, cause mutations. Detoxification and DNA repair depend on specific factors that may be affected by genetic polymorphisms or mutations in genes involved in carcinogen metabolism, as observed in Fanconi anemia, a rare genetic disease caused by a deficiency in DNA repair mechanisms, with an increased risk of developing HNSCC (6).

Conventional primary treatments for HNSCC are lesion resection and radiotherapy for early stages of the disease, and chemotherapy for locally advanced disease. Biological and chemotherapeutic agents combined with radiotherapy have demonstrated high levels of efficiency in local control and patient survival (15). For patients with recurrent or metastatic tumors, therapeutic options include immune checkpoint inhibitors, platinum derivatives, fluorouracil (FU) and cetuximab (CTX) (16). CTX is a monoclonal antibody that competitively targets the extracellular domain of EGFR, blocking proliferative, antiapoptotic and proangiogenic signals (17) Other EGFR inhibitors have been developed; however, they have not significantly improved the rates of patient survival, and are associated with high levels of toxicity (18).

Chemotherapy is one of the most commonly used treatments. However, drug resistance is a major obstacle in controlling disease progression. Tumors may be nonresponsive to a particular treatment due to intrinsic (or primary) resistance caused by inherited mutations or insensitive clones, whereas other tumors develop resistance (acquired or adaptative) after a positive response to therapy (14). Cells that survive initial chemotherapy represent the population reservoir from which resistant clones may emerge (15).

Several factors contribute to the development of drug resistance and understanding the mechanisms involved is crucial to improve cancer treatment. They include increased drug efflux, reduced drug uptake, drug inactivation, resistance to apoptosis, efficient DNA repair, epigenetic changes that affect gene expression, drug target mutations, overexpression or amplification of genes associated with resistance and genomic instability (19). Underlying these mechanisms are specific biological processes that are critical to drug resistance. For instance, selective protein degradation was shown to regulate the expression of three ATP-binding cassette transporters that limit drug uptake by cells (20). Evasion of apoptosis may equally be regulated by protein degradation and noncoding RNA or DNA demethylation through the downregulation of proapoptotic proteins (21,22).

Extrinsic factors, such as tumor microenvironment components, can also promote resistance. Su *et al* (23) showed that a carcinoma-associated fibroblast subpopulation that exhibits two cell-surface markers, membrane metalloendopeptidase (neprilysin) and complement C5a receptor 2 (C5AR2), provides a survival niche for cancer stem cells, which are tumorigenic and chemoresistant in certain cancer types. In addition, neprilysin^{+}C5AR2^{+} fibroblasts are resistant to chemotherapy and can induce chemoresistance in tumor cells by secreting interleukins IL-6 and IL-8.

Thus, chemotherapy resistance poses a complex challenge in cancer treatment. The present review aimed to address the potential relationship between tumor evolution, epithelial-mesenchymal transition (EMT), EGFR signaling alterations and drug resistance in HNSCC, with a focus on CTX chemotherapy.

2. Tumor evolution

Regarding the clinical evolution of HNSCC tumors, initial oral cavity lesions present as a painful mass or ulcer that compromises eating or speaking. Oropharyngeal and hypopharyngeal tumors are usually diagnosed at later stages with symptoms of dysphagia, odynophagia or otalgia, and HPV-positive cases may remain asymptomatic for numerous years. Early laryngeal manifestations include change in voice or hoarseness, but after evolving to a more advanced stage, they may compromise airway patency, leading to dyspnea. Clinical manifestations of nasopharyngeal tumors with EBV infection are stage-dependent, and initial cases may present with a solitary, painless cervical mass and unilateral nasal obstruction, which evolve to display other more severe symptoms, including otalgia, epistaxis, vision changes, headache and persistent rhinorrhea (6,24).

The evolution of the tumor mass is gradual. For numerous years, the progression of genetically transformed cells to a malignant condition was considered an isolated process. During this process, early neoplastic lesions recruit and activate stromal cells, such as fibroblasts, pericytes and adipocytes, as well as immune cells, to promote a microenvironment favorable to disease progression. These cells then alter the tumor microenvironment through the secretion of factors that maintain proliferative signaling and activate invasion and metastasis, ultimately favoring tumor cell homeostasis (25,26). In addition to biological agents, physical factors may alter the extracellular matrix, cytoskeleton and blood vessel permeability. These factors may also affect genome integrity and gene expression or localization of effectors that control the cell cycle, such as CDKN1B, NF-κB p105 subunit and transcriptional coactivator YAP1/WW domain-containing transcription regulator protein 1, contributing to neoplastic transformation, EMT and tumor evolution from abnormal growth patterns to increased tissue volume (27,28).

Regarding neoplastic cells, malignant transformation occurs through successive mutations that result in acquired capabilities or hallmarks. Hanahan and Weinberg (29) defined the hallmarks of cancer, which include sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, sustained angiogenesis, invasion and metastasis. A decade later, Hanahan and Weinberg (30) proposed two additional interrelated emerging hallmarks: Reprogramming of energy metabolism and evasion of immune destruction, which depend on genome instability and tumor-promoting inflammation, like other hallmarks. Recently, Hanahan (31) detailed the addition of two new hallmarks-unlocking phenotypic plasticity and nonmutational epigenetic reprogramming- and two enabling characteristics or 'facilitators' for the acquisition of hallmarks-senescent cells and polymorphic microbiomes.

Underlying the hallmarks are driver mutations, which are genomic alterations that confer growth or a survival advantage to the cell. Unlike driver mutations, passenger mutations have no role in the malignant phenotype and may be lost during the neoplastic process, but contribute to the tumor mutational burden, which is a potential biomarker of response to therapy (32).

Bypassing cell cycle checkpoints and increasing proliferation are achieved by synthetizing growth factors or receptors and inducing neighboring cells to produce ligands of interest. A similar effect is also attained through structural changes and amplifications of receptor molecules or through downstream effectors that maintain signals without requiring external stimuli (26). Further mutations modify the tumor microenvironment and allow tumor cells to pass through the extracellular matrix and reach other tissues. The overexpression of cytokines and growth factors promotes inflammatory infiltration and growth of blood and lymphatic vessels. The acquisition of such characteristics occurs through gene mutations and epigenetic modifications, as well as Darwinian selection, which drives the evolution of a healthy cell to a malignant population, allowing adaptation to new environmental pressures and progression to novel phenotypes (33).

Nowell (34) stated that cancer arises from a single cell and evolves linearly through the selection of mutations, resulting in a homogeneous clone with a strong selective advantage (Fig. 1A). Dexter *et al* (35) and others (36,37) have proposed a model in which tumors evolve in a branched fashion. According to this model, distinct mutated clones derived from the same clone are differentially selected by endogenous and external factors, which results in high intratumor heterogeneity (Fig. 1B). Conversely, Williams *et al* (38) proposed that alterations responsible for the neoplastic process are present in the first malignant cell and the subsequent mutations are neutral (Fig. 1C). Results of Williams *et al* (38) revealed that carcinomas of the stomach, lung and cervix display neutral evolution; pancreatic and thyroid carcinoma and glioblastoma display profiles compatible with nonneutral evolution, and HNSCC displays mixed evolution (Table I).

In nonneutral evolution, continuous clonal selection and adaptation to microenvironment niches have important roles in tumor progression. Groups of premalignant cells that undergo clonal expansion following chronic exposure to environmental carcinogens exhibit a high risk of generating multiple local primary tumors, a process that has been reported in various cancer types, including HNSCC. This process is known as field cancerization and may provide an explanation for the presence of tissue areas positive for p53 on immunostaining, prone to malignant transformation and with high rates of recurrence (39). In addition, studies on yeast or bacteria have revealed that neutral synonymous mutations in protein-coding genes, which do not change protein sequences, may affect mRNA structure, level or function and alter growth rates, particularly under strong selection (40,41).

Therefore, although Williams *et al* (38) and Li *et al* (42) have shown that a significant number of neoplastic subclones undergo neutral evolution, new biological or physical selective pressures, such as therapy, extracellular matrix remodeling or tissue structure, may promote the expansion of neutral clones, resulting in EMT, metastasis and tumor progression (28).

Studies on cancer biology have identified various single-nucleotide mutations and small insertion or deletion driver mutations that may be acquired over time. However, several tumor types exhibit complex chromosome aberrations that involve localized (chromothripsis) or dispersed (chromoplexy) genomic regions (43-46), which define a punctuated type of evolution (33) (Fig. 1D; Table I). These catastrophic events occur early in the neoplastic process, resulting in a rapid 'big bang' clonal expansion, which is characterized by uncontrolled cell proliferation (47,48). This instability is often unfavorable and may halt or even reverse tumor growth through selective pressures intrinsic to cellular metabolism or part of the immune response, as well as through factors from the tumor microenvironment (30). However, if successful, instability may lead to the development of aberrant cells that drive metastasis and therapeutic resistance (49,50).

By combining the evolutionary view of cancer with oncogene and tumor suppressor gene concepts, Fearon and Vogelstein (51) proposed a colorectal carcinogenesis model in 1990, in which mutations build linearly step-by-step. This sequence of events was later investigated in numerous other cancer types. Recent pan-cancer next-generation sequencing data have provided insight into intratumoral heterogeneity, which has a role in multiple shared evolutionary trajectories associated with prognostic biomarkers (52). In

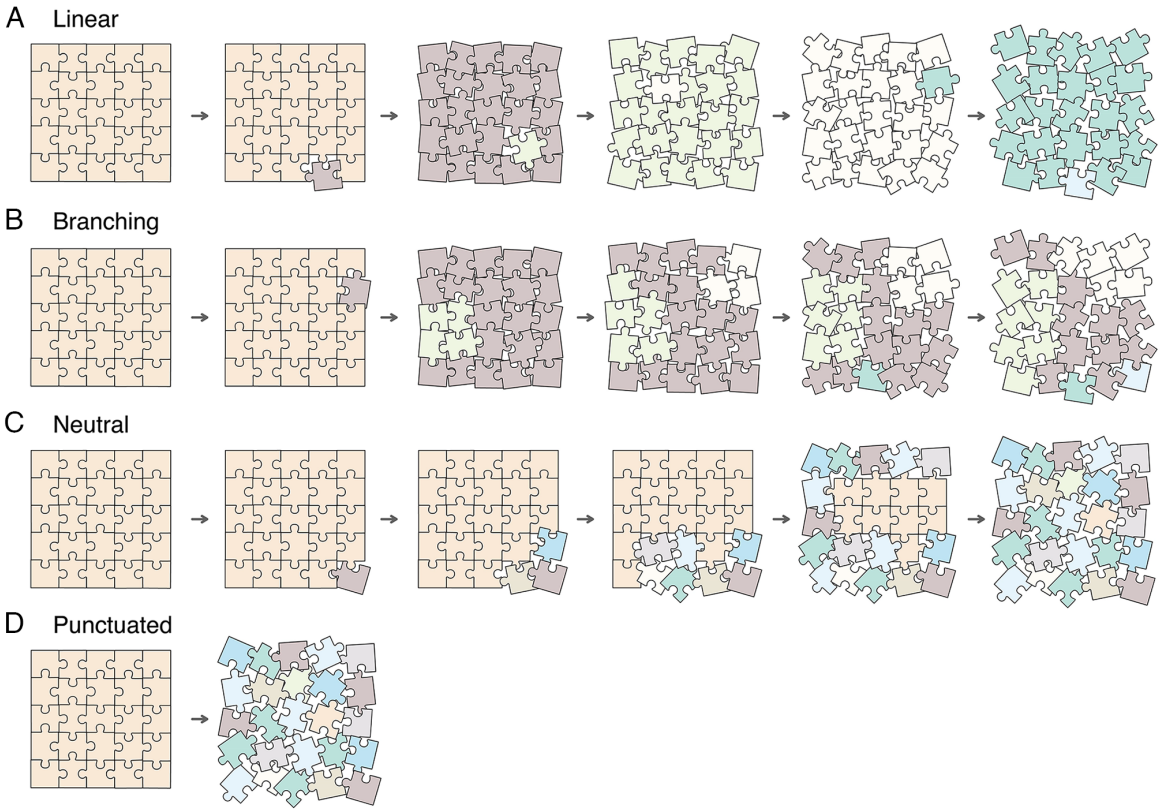

Figure 1. Models of Tumor Evolution. (A) Cancer arises from a single cell and evolves linearly through the selection of mutations, resulting in a homogeneous clone with a strong selective advantage. (B) Tumors evolve in a branched fashion, with distinct mutated clones derived from the same clone but differentially selected by endogenous and external factors, resulting in high intra-tumor heterogeneity. (C) The alterations responsible for the neoplastic process are present in the first malignant cell and the subsequent mutations are neutral. (D) Several tumors exhibit complex chromosomal aberrations that occur early in the neoplastic process.

certain cases, convergent evolution is observed, with distinct mutations acquired in the same gene or pathway (49,53,54). By contrast, other tumors show no evidence of subclonal driver mutation enrichment, but exhibit passenger mutations, which are not associated with cell growth and survival, but may have a role in intratumoral heterogeneity (49). Martínez-Jiménez *et al* (55) reported that metastatic lesions exhibit low intratumoral heterogeneity, which is consistent with the findings of Nguyen *et al* (56). These results were observed in numerous tumor types, including head and neck carcinomas, and may be explained by a dominant clone in the seeding event or selective external pressure.

Dynamic evolution associated with resistance to chemotherapy occurs in parallel to cancer evolution, generating adaptive costs for neoplastic cells in the form of vulnerability windows, known as persistent or temporary collateral sensitivity (57). This condition is also demonstrated in *Plasmodium* and bacteria and is based on the observation that a trait, such as drug resistance, may occur in detriment to another trait (58). The vulnerability window presents opportunities for new therapeutic approaches, combining the identification of tumor propensity toward resistance and sensitivity during tumor evolution.

These findings highlight the clinical relevance of tumor evolution model studies, as the mechanisms discussed may contribute to further understanding the complexities of cancer treatment. Therefore, identifying the driver markers of aggressive clones that proliferate under therapeutic and biological or physical pressures is important because they can predict resistance (59). It is important to identify subsequent metabolic reprogramming that supports a stress condition capable of selecting new mutations responsible for conferring higher phenotypic variability and adaptation (60).

In addition to genetic mechanisms, including mutations and chromosome aberrations, cancer cells can change from one phenotype to another through epigenetic and transcriptional adaptive processes without genomic alterations. These nongenetic mechanisms of cancer evolution may culminate in transdifferentiation to a dissimilar subclone and reversion to a progenitor phenotype or EMT, and represent a new challenge in the prediction and treatment of cancer resistance (61).

3. EMT

EMT is a cellular differentiation program and a phenotypic shift that culminates in the dissolution of epithelial cell-cell-contacts, such as desmosomes, tight junctions, adherens junctions and gap junctions, through the disruption of the Crumbs, partitioning-defective (PARD) and Scribble polarity complexes. Consequently, cells lose apical-basal polarity and epithelial traits (62). Expression of the epithelial cell adhesion protein E-cadherin is downregulated and expression of mesenchymal proteins, including N-cadherin, vimentin and fibronectin, is activated (63,64). The subsequent reorganization

Table I. Models of Cancer Evolution: Linear, branching, neutral and punctuated (refs. 33-48).

Evolution type	Mechanism of evolution	Intratumor heterogeneity	Examples
Linear	Genetic alterations successively selected step-by-step resulting in a major dominant clone	Low	Acute lymphoblastic leukemia
Branching	Co-existence and continued evolution of multiple subclones derived from a common ancestor	Variable	Breast, liver, colorectal and prostate cancers
Neutral	Acquisition of driver followed by random fixation of neutral mutations through genetic drift	High	Stomach, lung and cervical carcinomas
Punctuated	Gradual acquisition of driver mutations interspersed with rapid clonal expansion	High	Prostate cancer
Mixed	Shift from one to another evolution type or multiple types simultaneously	High	Head and neck carcinoma

of the epithelial actin microfilaments allows for motility through membrane projection, actin contraction and adhesion.

EMT is required for morphogenesis during embryonic development. The resulting mesenchymal lineage exhibits migratory properties that allow them to be recruited to specific sites in the embryo, where they undergo mesenchymal-epithelial transition (MET) and form new epithelial tissues (65). When aberrantly activated, EMT and MET contribute to neoplastic progression by allowing cells to migrate to other organs through lymphatic or hematogenous dissemination and metastasize. Due to intratumoral heterogeneity, different levels of EMT activation occur according to the area of the tumor mass, resulting in partial EMT (p-EMT) or total EMT (66). This may be explained by nonmutational epigenetic plasticity. In oral squamous cell carcinomas, the invasive front demonstrates p-EMT, with a loss of transcription factors directly involved in EMT, and the expression of other EMT-defining genes, which are absent in the central core of the tumors. This heterogeneity has not been associated with the presence of mutations in different tissue areas and may be attributed to epigenomic variability resulting from histone modification, DNA methylation and posttranscriptional modification of RNA (31). However, paracrine p-EMT regulation by microenvironmental stroma cannot be excluded (67).

Results of *in vitro* studies have demonstrated that cell lines may display different rates of p-EMT states and varying EMT phenotypes. Many exhibited a more epithelial phenotype, whereas others appeared to be mesenchymal with a higher migration rate (68). Cell migration consumes high levels of ATP and mesenchymal phenotypes may increase aerobic glycolysis. DeCamp *et al* (69) and others (70,71) focused on a specific process that promotes the acquisition of a plastic phenotype by epithelial cells, allowing them to perform collective epithelial-cell migration through mechanisms other than EMT, known as epithelial unjamming. This leads to a fluid-like migratory phase and a shift toward glycolytic energy metabolism without cell-cell junction disruption. It involves lipid, cellular ketone and carbohydrate metabolism, with the oxidation of fatty acids for ATP or energy generation in mitochondria. The authors hypothesized that epithelial unjamming may be an adaptation that allows the confluent epithelial collective to perform dynamic events, such as those occurring during embryonic development, wound healing and neoplastic progression, at the cost of high energy expenditure.

EMT is associated with stimuli in the tumor microenvironment and signaling pathways, including Wnt/β-catenin, NOTCH, transforming growth factor (TGF)-β/SMAD, PI3K/AKT and JAK/STAT, in which ligands and receptors, such as IL-6 (72,73), TNF (74), TGF, bone morphogenetic proteins (62,75) and tyrosine-kinase receptors (75), interact. These signaling pathways regulate the expression of transcription factors and their epithelial targets, such as E-cadherin, and mesenchymal targets, such as N-cadherin, vimentin, fibronectin and MMPs/metalloproteinases. Numerous genes are associated with EMT. The EMT gene database (dbEMT; http://dbemt.bioinfo-minzhao.org/; version 2.0) lists 1,184 EMT-related genes, 1,011 protein-coding genes, and 173 noncoding RNAs (57). However, few genes are directly associated with EMT, such as the transcription regulators zinc finger protein SNAI1 (SNAI1), zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2 and twist family bHLH transcription factor (TWIST), which bind to specific DNA sequences in promoters or enhancers through zinc finger domains, homeodomains or helix-loop-helix domains that activate the transcription of target genes (76).

SNAI1 and ZEB1 induce EMT by binding to the promoter of the E-cadherin gene (cadherin 1; *CDH1*) and the promoters of *MMP2* and *MMP9*, repressing and promoting their transcription, respectively. ZEB1 also stimulates the expression of SETD1B (histone-lysine *N*-methyltransferase SETD1B), a histone methyltransferase that influences chromatin organization, in addition to a positive feedback loop with ZEB1. Similarly, upregulation of SNAI1 causes alterations in the chromatin state, which may consequently activate EMT genes. TWIST modulates mesenchymal and epithelial phenotypes, thus regulating the transcription of both N-cadherin/*CDH2* and E-cadherin/*CDH1*, respectively (76) (Fig. 2).

The contribution of SNAI1, ZEBs and TWIST to EMT is subordinated to the cell type and the presence of activating ligands and stimuli in the tumor microenvironment (75). For instance, Puram *et al* (67) did not detect the expression of classical EMT transcription factors in an analysis of the HNSCC

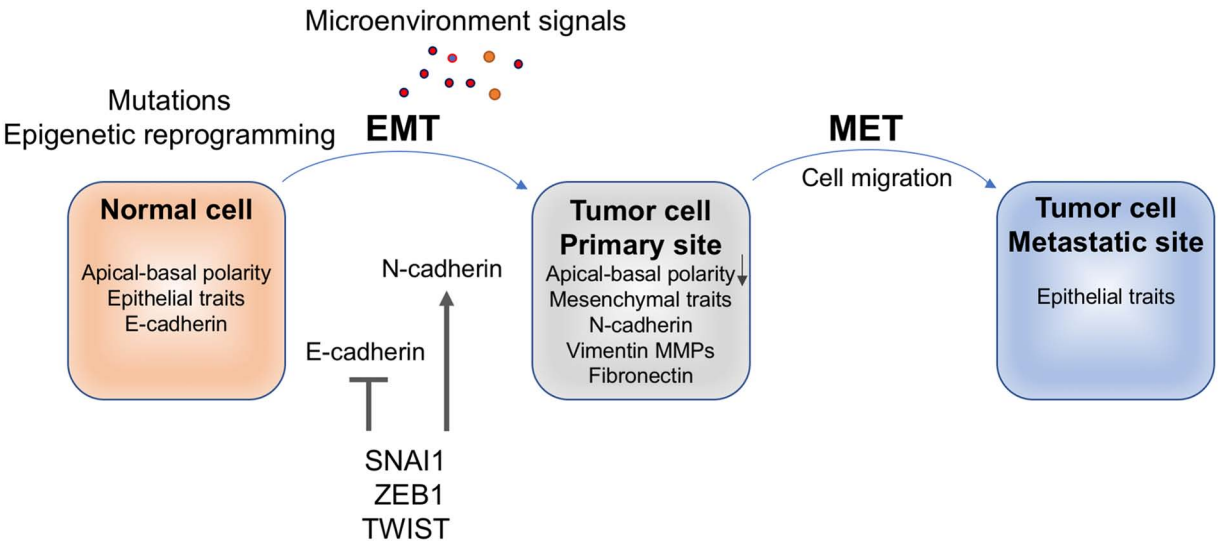

Figure 2. EMT in Neoplastic Development and Metastasis. *EMT* is characterized by loss of apical-basal polarity, low expression of epithelial markers (such as E-cadherin) and expression of mesenchymal markers (including N-cadherin, vimentin, fibronectin and metalloproteinases/MMPs). The mesenchymal lineage exhibits migratory properties that allows cells to invade and metastasize. Once in a new niche, they are able to undergo MET and dedifferentiate to epithelial tissues. MET, mesenchymal-epithelial transition; EMT, epithelial-mesenchymal transition; MMP, matrix metalloproteinase; ZEB1, zinc finger E-box binding homeobox 1; TWIST, twist family bHLH transcription factor.

single-cell transcriptome. However, results of the present study demonstrated that most epithelial and mesenchymal EMT markers, including vimentin, integrin, TGF-β-induced genes and SNAI2, were maintained. Thus, Puram *et al* (67) hypothesized that the p-EMT state localized at the leading edge of the tumor is distinct from the full EMT state and may be due to paracrine interactions between stromal and neoplastic cells. Puram *et al* (67) also determined the potential association between high p-EMT and number of lymph node metastases, tumor grade and adverse pathological characteristics, including extracapsular extension and lymphovascular invasion. Of note, overexpression of factors involved in EMT has been associated with metastasis, advanced stages of disease and low overall survival rates in patients with various cancer types (76,77), and were confirmed in HNSCC (78,79). Okuyama *et al* (80) highlighted the association of tumor budding (defined as the presence of isolated clusters of up to five cancer cells ahead of the invasive tumor front) with p-EMT, and determined the potential of tumor budding as a prognostic marker for poor survival in HNSCC. Therefore, these biomarkers are potential targets for cancer treatment.

4. EGFR and HNSCC

Receptor tyrosine kinases (RTKs) are key regulators of EMT. EGFR is an RTK that has an important role in cell physiology by regulating several signaling cascades involved in critical cellular functions such as proliferation, differentiation, survival and motility. However, when EGFR is mutated or overexpressed, it can trigger a neoplastic process (81).

EGFR is a transmembrane glycoprotein and a member of the ErbB tyrosine-kinase family encoded by *EGFR/ERBB1/HER1* at 7p11.2 [gene ID, 1956; National Center for Biotechnology Information (NCBI), https://www.ncbi.nlm.nih.gov/gene/1956]. EGFR possesses an extracellular ligand-binding region and a cytoplasmatic kinase domain connected by a transmembrane helix. The extracellular region of EGFR has four domains: I and III are leucine-rich domains for ligand binding, while II and IV are cysteine-rich domains. Binding of the EGFR ligands, such as EGF, amphiregulin, betacellulin, epigen, epiregulin heparin-binding EGF and TGF-α (82) promotes a conformational change from a self-inhibited 'tethered' state to an open 'untethered' state. The new conformation facilitates domains II and IV to bind to an adjacent RTK and homo- or heterodimerize, inducing auto-phosphorylation, activation and recruitment of effectors to initiate a signaling cascade (83,84). Purba *et al* (85) proposed that EGFR is an inactive dimer prior to ligand-induced dimerization. When the ligand binds the receptor, its transmembrane sequence may undergo a rotation, resulting in an active configuration of the intracellular domain of the RTK. Hence, mutations in the extracellular sequence may promote the rotation and activation of the receptor without the ligand (84) (Fig. 3).

In 232 HNSCC cases analyzed in the TCGA HNSC project, 17 (7.33%) somatic mutations and no copy number variation gains or losses were observed in *EGFR*. However, upregulation of *EGFR* and associated ligands is frequent in HNSC (6,86), which may be a result of the expression of inflammatory mediators and transcription factors caused by external factors, such as tobacco smoke. G protein-coupled receptor signaling (82) activation by p53 protein, polymorphisms in intron 1 of *EGFR*, and *EGFR* amplification (85,87) are alternate mechanisms that increase *EGFR* expression or synthesis of associated ligands. Kriegs *et al* (86) demonstrated that EGFR expression and autophosphorylation in HNSCC cells are not well correlated, and may be dependent on other factors, such as EGFR polymorphisms and mutations in downstream effectors.

Pleiotropic functions of EGFR in HNSCC and other tumors are associated with MAPK/ERK (proliferation, immunosuppression, angiogenesis) (88,89), PI3K/AKT (cell survival and proliferation; apoptosis evasion) (89,90),

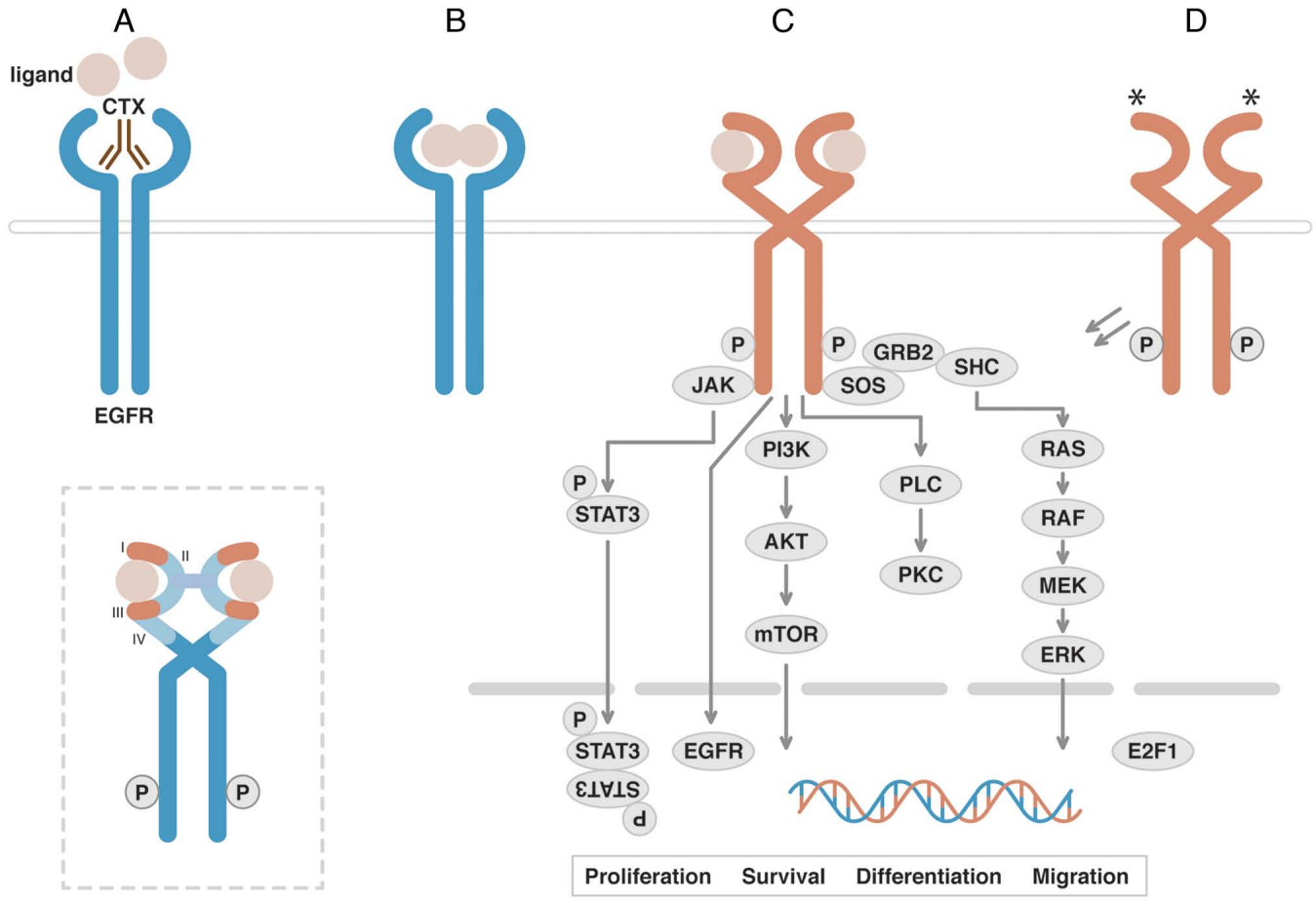

Figure 3. EGFR Signaling Transduction and Downstream Effectors. (A) EGFR is a receptor tyrosine kinase that possesses an extracellular ligand-binding region and a cytoplasmatic kinase domain connected by a transmembrane domain. CTX is a chimeric human/mouse monoclonal antibody that binds to domain III of the receptor blocking binding of natural ligands. (B) Binding of the EGFR ligands promotes a conformational change that facilitates homo- or heterodimerization, inducing (C) autophosphorylation, activation and recruitment of effectors to initiate a signaling cascade. (D) Mutations (*) in the extracellular sequence may promote the activation of the receptor without the presence of a ligand. The dashed box highlights a schematic diagram of extracellular ligand-binding domains (I and III) of EGFR. AKT, RAC serine/threonine-protein kinases; E2F1, transcription factor E2F1; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; GRB2, growth factor receptor-bound protein 2; JAK, Janus kinase; MEK, MAP kinase kinases; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase-C2-α; PKC, protein kinase C; PLC, phospholipase C-γ-1; RAF, proto-oncogene c-RAF; RAS, GTPase-activating protein; SHC, SHC-transforming protein 1; SOS, son of sevenless homolog 1; STAT3, signal transducer and activator of transcription 3; I, II, III, IV, EGFR extracellular domains.

JAK/STAT (cell growth, development, differentiation and survival) (91) and phospholipase (PLC) C-γ-1/protein kinase C (PKC) (proliferation, migration) (92,93). Particularly when mutated or overexpressed in HNSCC, EGFR has protumorigenic and prometastatic roles associated with nutrient uptake and biosynthesis, proliferation, inflammation, cell survival, migration and invasion. In addition to the membrane-bound functions, EGFR activation may result in its endocytosis and translocation to the nucleoplasm, where it modulates the activity of numerous genes, including those associated with the cell cycle, such as *CCND1*, DNA damage response, e.g. the catalytic subunit of the DNA-dependent protein kinase, DNA replication (e.g. proliferating cell nuclear antigen, cofactor of DNA polymerase), mitochondrial electron transport, such as cytochrome c oxidase subunit II, and inflammation, e.g. nitric oxide synthase 2 (94).

Multiple strategies have been developed to inhibit EGFR in HNSCC, such as monoclonal antibodies that compete for the ligand-binding region to prevent the activation of the cytoplasmatic tyrosine kinase domain and small inhibitor molecules that bind to the kinase domain to block EGFR autophosphorylation and downstream signaling. CTX, zalutumumab, panitumumab and nimotuzumab are examples of anti-EGFR antibodies, and gefitinib, erlotinib and lapatinib are tyrosine kinase inhibitors. CTX is approved by the Food and Drug Administration as an anti-EGFR agent for use in HNSCC chemotherapy. Several studies have detailed the associated efficacy and adverse events (95-97).

5. CTX therapy, and head and neck carcinoma

The majority of head and neck carcinoma cases are diagnosed at late stages and thus associated with short progression-free survival. Current treatment options aim to improve overall survival and delay disease progression while maintaining quality of life. The current conventional treatment involves surgical intervention and lesion irradiation for early-stage cases and chemotherapy for advanced cases. The results of a meta-analysis involving 107 randomized trials and 19,805 patients demonstrated that adjuvant chemotherapy

following primary treatment involving surgical lesion removal or radiotherapy did not increase overall survival. Furthermore, certain levels of toxicity were observed. By contrast, concomitant chemotherapy for locally advanced disease exerted positive effects on overall survival. However, in patients aged ≥70 years, overall survival rates were markedly decreased following concomitant chemotherapy; thus, treatment options should be discussed thoroughly with multidisciplinary health teams and patients' families (98).

Platinum derivatives with FU + CTX are considered the standard of care chemotherapy treatment for recurrent or metastatic HNSCC. This treatment option (EXTREME protocol) significantly increased the median overall survival of patients from ~7 months in the chemotherapy-alone group to ~10 months in the group that received chemotherapy plus cetuximab (99-101). Cisplatin is an inorganic platinum derivative that induces DNA intrastrand crosslinks, subsequently interfering with DNA replication and transcription, leading to cell-cycle arrest and apoptosis (102). FU, a fluorinated pyrimidine analogue, causes cellular cytotoxicity through a complex metabolic process. For instance, fluorodeoxyuridine triphosphate can be incorporated into nucleic acids affecting DNA and RNA functions, whereas the metabolite fluorodeoxyuridine monophosphate, resulting from the conversion of FU by thymidine phosphorylase and thymidine kinase, forms a stable complex with thymidylate synthase, resulting in thymidine depletion, decreased DNA synthesis and cell lethality (103).

CTX is a chimeric human/mouse immunoglobulin G1-subclass monoclonal antibody drug that binds to the extracellular ligand-binding region (domain III) of EGFR (Fig. 3A) with higher affinity than the natural ligands EGF and TGFα. CTX blocks the untethering conformation of the receptor monomer, further inhibiting dimerization, eventually halting the activation of the tyrosine kinase domain and proliferation signaling through RAS and ERK (104,105).

Other antitumor mechanisms of CTX are inhibition of ligand-receptor binding, internalization and degradation of EGFR, and antibody-mediated cytotoxicity (106). CTX also activates proinflammatory and proapoptotic factors and inhibits repair of radiation-induced lesions, increasing the efficacy of strategies that combine EGFR-targeted agents and immune- or radiotherapy. CTX blocks invasion, angiogenesis and metastasis, which are protumorigenic roles of several signaling pathways associated with EGFR (81,107). By contrast, activating mutations in members of the RAS pathway may reduce the efficacy of CTX, leading to an improper ERK response and changes in transcription, cell fates and proliferation (108).

Jie *et al* (109) observed that in HNSCC, CTX therapy increased intratumoral regulatory T cells with an immunosuppressive phenotype, but promoted impaired expression of molecules related to antibody-dependent cellular cytotoxicity of natural killer cells in the tumor microenvironment. This inverse correlation indicates that regulatory T cells restrain infiltrating natural killer cell-mediated cytotoxicity, which negatively affects CTX therapy in this group of tumors. CTX also exerts synergistic effects, such as increasing radiation-induced apoptosis by blocking DNA repair mechanisms dependent on PI3K/AKT, MAPK/ERK and JAK/STAT pathways (104).

Different mechanisms of action of CTX were also observed in HNSCC, such as upregulation of the transcription factor encoded by transcription factor AP-2α (*TFAP2A*) by Kagohara *et al* (110). Their results suggested that *TFAP2A* induces cell proliferation and is potentially overexpressed by CTX to overcome EGFR inactivation. The authors also detected upregulation of the *AXL* gene, which encodes a receptor tyrosine kinase related to growth, migration and inflammation, and overexpression of several EMT genes of collagenases.

As CTX is a large monoclonal antibody, it cannot be filtered by the kidneys. Thus, a small fraction is eliminated by biliary excretion, and the majority is eliminated through intracellular catabolism (111). CTX may be administered alone in cases eligible for radiotherapy when platinum-based therapy is not appropriate (112). Contraindications to receiving cisplatin consider factors such as the Eastern Cooperative Oncology Group Performance Status score, age, comorbidities, involuntary weight loss, concomitant medications and prior platinum-based chemotherapy (112-115).

Alternate regimens were developed to enhance the overall and progression-free survival rates in patients with HNSCC. For instance, the TPEx protocol, which combines the taxane docetaxel, CTX and cisplatin, increased median overall survival to 14 months (116) and improved tolerance to treatment and quality of life (100), compared with the EXTREME protocol. In patients with HPV and oropharyngeal cancer, results of the De-ESCALaTE HPV trial demonstrated that CTX exerted no benefits in toxicity or tumor control compared with radiotherapy plus cisplatin (117). This result is expected, because HPV-positive cases of HNSCC are associated with the viral oncoproteins E6 and E7 rather than the EGFR protumorigenic signaling pathways targeted by CTX (118).

Primary or intrinsic resistance to CTX may occur in a small number of patients with HNSCC; however, almost all patients develop acquired resistance. Intrinsic mechanisms of resistance include alterations in EGFR and the associated ligands or effectors, acquired mutations in genes of alternative oncogenic pathways involved in tumorigenesis, such as Ras/Raf/MAPK/ERK and PI3K/AKT/mTOR, loss of phosphatase and tensin homolog (PTEN) or phosphorylation of STAT3, and epigenetic modifications. In contrast to intrinsic resistance, mechanisms associated with acquired resistance in HNSCC are diverse and include alterations in EGFR and its ligands, activation of the PI3K/AKT/mTOR signaling pathway, loss of PTEN, EMT phenotype acquisition, phosphorylation of STAT3, epigenetic alterations and an immunosuppressive tumor microenvironment (104,119-122). For example, EGFR mutations in subdomain I or close to the EGF-binding pocket (G33S and N56K) promote an open untethered receptor with a reduced affinity for EGF and CTX, but with decreased degradation and sustained activation of AKT signaling (84). The literature on CTX resistance in HNSCC has also reported abnormal expression of markers directly associated with EMT, including vimentin (110), ZEB2, TWIST1, SNAIL, E-cadherin and fibronectin, and markers related to EMT process, such as MAD homolog 4, main components of the epithelial cytoskeleton (keratins 13, 14 and 16), and the metalloproteinase ADAM 19 (121,123,124), as well as alternate mechanisms of resistance or sensitivity associated with microRNA-9 (125) and polymorphisms of cytochrome P450 1B1 (126).

Using high-throughput screening to examine the activity of 42 RTKs, Wheeler *et al* (127) reported that EGFR, HER2 and HER3 are highly activated in CTX-resistant HNSCC cells. The mechanisms involved comprise a dysregulation of EGFR internalization or degradation and EGFR-dependent activation of HER2 and HER3, which initiate a proliferative and survival signaling cascade.

In addition to the HER family and the downstream effectors, EGFR blockade in HNSCC results in the activation of several alternative growth factor receptor pathways, such as anaplastic lymphoma kinase (ALK), insulin-like growth factor (IGF)-1 and hepatocyte growth factor (MET), which are members of the RTK family (128). MET activation modulates several signaling cascades, including PI3K/AKT, JAK/STAT, Ras/MAPK, SRC and Wnt/β-catenin (129). MET has been found to be increased in lymph node metastases of HNSCC, compared with primary tumors (130). IGF-1 receptor (IGF-1R) is associated with tumorigenesis of epithelial cancers. Its activation potentially requires EGFR kinase activity or IGF-1R/EGFR complex formation, and stimulates G1- to S-phase transition in a PI3K/AKT and ERK-dependent pathway (131). ALK is a marker upregulated in advanced HNSCC, compared with early-stage tumors (132). Functional assays and evaluation of receptor expression or activation and mutational status of effectors indicate that MET and ALK activation, as well as increased heterodimerization of EGFR and IGF-1R, are mechanisms of CTX resistance in HNSCC (133-135).

Umemori *et al* (136) observed increased expression of epithelial cell adhesion molecule (Ep-CAM) products in HNSCC samples. Ep-CAM is a transmembrane glycoprotein that promotes cell adhesion, cell proliferation, EMT and cancer stemness, and is a potential prognostic marker for human carcinomas. Its N-terminus (EpEX) exhibits EGF-like domains that can function as ligands for EGFR (137,138). Umemori *et al* (136) showed that EpEX competes against CTX and stimulates the EGFR-ERK pathway, contributing to resistance.

Furthermore, an energetic metabolic shift during treatment has been observed. Results of a study using HNSCC patient-derived tumor xenografts revealed that the lactate to pyruvate ratio was significantly decreased in CTX-sensitive xenografts between pretreatment and posttreatment, but it was not affected in CTX-resistant xenografts (139). These results indicate that a glycolytic phenotype may contribute to the development of CTX resistance.

Several studies have suggested that resistance to anti-EGFR therapies is related to hypoxia and angiogenesis. Hypoxia occurs in conditions with high demands of oxygen due to increased proliferation rates and deficient angiogenesis These conditions induce the expression of hypoxia-inducible transcription factors (HIFs), which are responsible for upregulating genes involved in oxygen supply (e.g., in modulating angiogenesis) and genes that limit oxygen consumption (involved in nonoxidative metabolism). In tumor cells, HIFs also activate genes that promote EMT, immune evasion, reprogramming of energy metabolism and acquisition of cancer stem cell properties. Vascular endothelial growth factor and other angiogenic growth factors, such as stromal-derived factor 1, stem cell factor and angiopoietin family members, are also regulated by HIFs (140). As expected, EGFR pathway inhibition decreases HIF and vascular endothelial growth factor expression and renders the tumor sensitive to antiangiogenic therapies; thus, combining the two approaches may improve outcomes (141,142).

In patients with HNSCC, hypoxic tumors show a microenvironment with increased numbers of immunosuppressive regulatory T cells, a feature that may contribute to a deficient immunotherapy response. These tumors also show enrichment of hypoxia and EGFR and TGF-β signaling genes and decreased expression of interferon (IFN)α and IFNg effectors. A significant shift toward hypoxia normalization and growth of T- and B-cell subsets was observed after CTX therapy prior to surgery. These findings indicate that CTX combined with immunotherapy may overcome resistance in a group of patients with hypoxic tumors (107). An *in vitro* study demonstrated that HNSCC cell lines exhibit sensitivity to CTX under hypoxic conditions, inducing downregulation of HIF-1α and reduced growth. However, both HIF-1α suppression and CTX treatment were unable to inhibit EMT and reduce the expression of stem cell markers. Therefore, at least *in vitro*, resistance to CTX in HNSCC cells appears to be independent of hypoxia conditions (143).

Ge *et al* (144) demonstrated that in patients with HNSCC treated with CTX, dynamic changes in the levels of T-cell receptors in peripheral blood and tumor tissue occur, which may be associated with therapeutic responses. These results highlight the importance of evaluating T-cell receptors and determining their potential use as a noninvasive approach for assessing response to CTX in HNSCC. In addition, monitoring clonal composition and circulating molecules, while analyzing three-dimensional systems, such as patient-derived organoids and organotypic culture, may aid in understanding tumor progression and therapeutic resistance mechanisms (104,122,145).

Resistance to therapy is a major task in cancer treatment and occurs through numerous routes: Drug targets acquire mutations or are downregulated; alternative pathways are activated; drug pumps are upregulated; xenobiotic receptors, detoxicating enzymes and efflux transporters are downregulated; metabolic profiles of the tumor and its microenvironment are changed; apoptosis resistance and immune evasion are stimulated; and DNA repair is altered. Extrinsic factors include hypoxia, inflammation, immune response, and other microenvironment characteristics. Many solutions for this scenario can be proposed, such as more specific drugs, combined and sequential therapies, synthetic lethality and immunotherapy (146). Gene editing using clustered regularly interspaced short palindrome repeat technology is a useful tool for identifying genes and signaling pathways that participate in cancer drug resistance and providing an alternative for removing resistant cells (147).

Slow progress in the field of therapy resistance is a challenge because tumor heterogeneity, another variable that cannot be eliminated, remains an efficient barrier underlying each attempt to address a negative event. The tumor microenvironment is heterogeneous with regard to the cell types, available activators or repressors, and stromal structure and vessels. Cancer stem cells may use different mechanisms to escape from foreign molecules or conditions. The tumor

itself contains dissimilar subpopulations that can generate clones with new characteristics, many of which are selectively neutral, but some have phenotypic advantages that can result in high heterogeneity. The cells themselves may use alternative signaling pathways for the same stimulus or *vice versa*. Ultimately, therapy triggers heterogeneity, which in turn paves the way for the development of therapy resistance, forming a vicious circle.

However, tumor heterogeneity has an Achilles' heel. A high mutational burden is potentially translated into neoantigens, which are targets for immunotherapy. Although immunotherapy has proved to be a valuable approach to treat different cancer types, including HNSCC, it induces drug resistance (148) and is limited to tumors showing appropriated levels of immunogenicity. Nanomedicines promise to circumvent this limitation, improving, for example, the release and presentation of tumor antigens in cases with low immunogenicity (149,150). Tissue editing approaches, which reprogram cancer hallmarks into biologic hallmarks, may also bring new tools to circumvent tumor heterogeneity and therapy resistance by using continuous low-dose chemotherapy for inducing stress responses, and transcriptional modulators for inflammation control (151).

6. Conclusions

Cancer is often considered a genetic disease. In the human body, genetic material is constantly changing and characteristics acquired through this process may lead to genomic instability and metabolic reprogramming of growth and survival. These changes may not be suppressed by healthy cells.

At present, therapeutic options available for heterogeneous tumor entities, such as HNSCC, are still limited due to a lack of understanding of the multitude of pathways involved. The development of novel methods to circumvent resistance and inhibit disease progression is required, aiming to kill as many cancer cells as possible, removing sensitive and retaining resistant cells (152).

Further investigations are required to classify the molecular landscape of HNSCC, and to evaluate relevant therapeutic resistance and prognostic markers. In addition, novel therapeutic options are required to obtain higher rates of patient survival and reduce the levels of associated toxicity and adverse events that may affect patient quality of life. Drug resistance studies provide valuable insight into the mechanisms underlying cell fate pathways and the neoplastic microenvironment. Research that utilizes evolutionary methodological approaches may further categorize metabolic contexts, changes in protein expression and function over the course of the disease, diagnosis and treatment.

In conclusion, recent technological advances have contributed to the understanding of the human genome and tumor evolution, and have led to the identification of numerous diagnostic and prognostic markers. However, many challenges remain within clinical practice, including high levels of tumor heterogeneity, undetectable clones in current assays, drug-resistant mutations that may be pre-existing or subsequent to therapy, identification of collateral vulnerabilities, a lack of distinction between tumor mutations and mutations in healthy aging cells, a lack of early disease detection and prevention, and a limited understanding of key mechanisms of drug resistance. Novel technologies and computational strategies coupled with further research and clinical trials may lead to improvements in the efficacy of cancer treatment (153-155).

Specifically regarding CTX resistance in HNSCC, the combined use of drugs partially overcomes tumor evolution inhibiting molecular signaling pathways crucial for the maintenance of cancer hallmarks. In fact, current data have identified several genomic expression profiles that correlate to resistance in HNSCC. However, successful laboratory-based studies do not translate into clinical applications. Novel drug delivery methods, reduced toxicity, tailored therapies and more randomized controlled trials are needed to develop more specific and efficient clinical approaches to improve survival and quality of life for patients with HNSCC.

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Authors' contributions

CHPD contributed to the study's conception and manuscript writing; TH, ACBS and TBC contributed to the study's conception. EHT contributed to study conception and manuscript-writing and review. All authors have read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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