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Understanding the Pathobiology of Head and Neck Squamous Cell Carcinoma

Chia-Cheng Li · Sook-Bin Woo

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Abstract Head and neck squamous cell carcinoma (HNSCC) is one of the leading cancers in the world, although wide geographical variations do exist. HNSCC can be subcategorized into conventional HNSCC and HPV-associated HNSCC, exhibiting distinct clinical and histopathologic features. Awareness of the risks of smoking has fortunately contributed to the decreasing incidence of conventional HNSCC in the USA. However, the prevalence of HPV-associated HNSCC in the USA has been significantly increasing. Much progress has been made in the research of development and progression of HNSCC. In this article, we review the current concepts of the pathobiological mechanisms of HNSCC.

Keywords Head and neck squamous cell carcinoma · Pathobiology · High-risk HPV

C.-C. Li (✉) · S.-B. Woo
Department of Oral Medicine, Infection, and Immunity, Harvard School of Dental Medicine, Boston, MA 02115, USA
e-mail: chia-cheng_li@hsdm.harvard.edu

S.-B. Woo
e-mail: swoo@rics.bwh.harvard.edu

C.-C. Li
Division of Genetics, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115, USA

S.-B. Woo
Division of Oral Medicine and Dentistry, Brigham and Women's Hospital, Boston, MA 02115, USA

S.-B. Woo
Center for Oral Pathology, StrataDx, Lexington, MA 02421, USA

Introduction to Head and Neck Squamous Cell Carcinoma

Cancer, a major cause of mortality, is a global health issue, and squamous cell carcinoma (SCC) of the head and neck constitutes 90 % of all cancer cases in the oral and oropharyngeal regions [1]. There is a wide geographic variation. Based on a study of cancer incidence in 2008, an estimated 263,900 new SCC cases only from the oral cavity and lips were newly diagnosed worldwide (170,900 cases in males, 64.8 %; and 93,000 cases in females, 35.2 %), with 128,000 deaths [2]. Oral SCC is the 8th leading cancer in men in the USA with an approximate annual incidence of 40,000 new cases and 8,000 deaths [3–7]. However in countries such as India and Pakistan, where there is a high prevalence of areca nut use, oral SCC represents the most common cancer in men [2, 8].

Multiple factors contribute to the initiation and progression of head and neck squamous cell carcinoma (HNSCC). Usage of areca nut, alcohol, and tobacco are well-recognized risk factors for SCC, both for the oral cavity proper and the oropharyngeal region [9]. Human papillomavirus (HPV) infection on the other hand, stands out as a risk factor for oropharyngeal SCC, with increasing incidence noted within the last 15 years [2, 10]. HPV-positive HNSCC exhibits a better prognosis than HPV-negative HNSCC for specific anatomic locations (e.g., tonsils), with a five-year survival rate of 89 % and 65 %, respectively [11]. Moreover, the median survival period is significantly longer in HPV-positive oropharyngeal SCC (91 months) compared to HPV-negative ones (76 months) [11]. In addition to the aforementioned exogenous risk factors, certain hereditary conditions, such as Fanconi anemia and Li-Fraumeni Syndrome, are predisposed to HNSCC [12]. The mainstay of treatment for HNSCC is surgery with adjuvant radiotherapy or chemo-radiotherapy for high stage disease with accompanying morbidity. The overall

five-year survival rate of conventional HNSCC is approximately 60 % [3, 7].

Targeted therapies are now used increasingly to manage cancers and improve survival rate. Such therapies specifically target crucial proteins within key pathways in the development of cancer. Although much progress has been made in the study of the development and progression of HNSCC, many specific mechanisms are still unclear. This article reviews the current concepts underlying the pathobiology of HNSCC.

HNSCC Initiation

Cancer Stem Cells (Cancer Initiating Cells)

HNSCCs cause significant morbidity and mortality, and may recur after surgical excision and adjuvant chemotherapy and radiotherapy [13]. This clinical fact has led to the cancer stem cell (CSC) model that hypothesizes the existence of a distinct cell population within HNSCC tumors, which may be resistant to the conventional therapy. HNSCC contains cellular heterogeneity, within which a developmental hierarchy exists [14]. CSCs or cancer initiating cells, consist of less than 10 % of the cells in an HNSCC tumor, and are able to self-renew and to aberrantly differentiate into heterogeneous cancer cell populations [15]. Identification and characterization of these CSCs will help us gain more insight in cancer biology, predict the tumor behavior, and develop better treatments to efficiently target this cell population.

Currently, flow cytometry, in vitro clonal trial, and serial transplantations are some methodologies used to detect CSCs. Several CSC-like markers (e.g., CD44; Bmi1; aldehyde dehydrogenase, ALDH; and CD133) have been identified, and the labeled cells demonstrated robust tumorigenic activity after serial transplantations [15–17]. Furthermore, evidence suggests that the existence of these labeled cells in oral SCC is associated with a poorer prognosis [18]. CD44 is associated with MMP9 activity [19], and the up-regulated expression of CD44 can potentially be utilized to determine tumor invasiveness and aggressiveness [20]. Bmi1 (B-cell specific Moloney murine leukemia virus insertion site 1) is a transcription repressor for cell senescence, implicated in the self-renewal of stem cells [21]. Bmi1 is highly expressed in the $CD44^+$ cell population sorted from oral SCC tumors, suggesting a potential role within CSCs [15]. ALDH labels a highly tumorigenic cell subset within the $CD44^+$ population [16]. Compared to the $CD133^-$ human oral SCC cells, the CD133 overexpressed cell population demonstrates stemness, invasiveness, and tumorigenic activity [17]. CD133 coordinates with the Src signaling pathway and contributes to epithelial-mesenchymal transition [17]. While multiple markers have been identified, and have shed light on the etiologic role of CSCs, the detailed signaling mechanisms are not yet fully understood.

Field Cancerization

The development of cancer through the entire upper aerodigestive tract is due to the accumulation of multiple genetic abnormalities via a multi-step process whereby cells in a particular tissue/organ are genetically altered but histologically normal, predating the development of neoplasia or coexisting with malignant cells [22–24]. As such, it is possible that precancerous lesions that already carry mutations may not even be visible clinically or abnormal histologically. This concept of "field cancerization" was proposed by Slaughter in 1953 to explain the high frequency of development of synchronous or metachronous HNSCCs [25, 26].

Microscopically, HNSCCs often have wider horizontal spread than infiltrative growth ("lateral cancerization") [24, 25]. In excised HNSCC specimens with clinically "clear" margins, hyperkeratotic, hyperplastic, or abnormally atrophic epithelium is often seen adjacent to the cancerous lesions, indicating that the lateral field is already "cancerized" [25, 26]. Within the "normal appearing" epithelium approximating the cancer, chromosomal aberrations (e.g., loss of heterzogosity, microsatellite alterations), and genetic/epigenetic mutations have been detected [26, 27]. Loss of heterzygosity (LOH) is the loss of one allele of a gene (e.g., tumor suppressor gene), such that if the remaining allele containing the other copy of the same gene becomes inactivated by a point mutation, this will result in the affected individual being more susceptible to cancer. LOH at the chromosomes 17p, 3p, 9p, 8p, 18q, and 11q has been associated with HNSCCs, and the order of occurrence as a progression model of HNSCCs has been proposed [26]. However, due to the lack of proper control and longitudinal follow-up, this model is not universally accepted. Nevertheless, it is likely as with cancers elsewhere in the body, that as a result of cumulative molecular events, CSCs transform to gain clonality and a selective proliferation advantage. The detailed pathologic mechanisms of HNSCCs will be discussed in the following sections.

Pathobiologic Mechanisms of HNSCC

To initiate a cancer, accumulated genetic and epigenetic alterations within an otherwise normal cell allows it to acquire the capabilities of becoming neoplastic and malignant. Six hallmarks of cancer were proposed by Weinberg and Hanahan in 2011 to conceptualize the initiation, progression, and dissemination of malignancies [28, 29]. Gene mutations leading to the up-regulation of oncogenes or down-regulation of tumor suppressor genes are seen in many cancers, including HNSCC. Epigenetic modifications are heritable changes that regulate gene activity through adjusting the structure and function of

chromatin, without affecting DNA code sequences, by processes such as DNA hypermethylation and post-translational histone modifications [30]. DNA hypermethylation, interrupting the binding of transcription factors at promoter regions and silencing of tumor suppressor genes (e.g., p16), is commonly detected in HNSCCs (50 %–73 %) [31, 32]. In addition, DNA hypermethylation of CDH1, MGMT, and DAPK in HNSCCs have been reported [31–33]. Interestingly, tissues adjacent to cancerous lesions also exhibit hypermethylation of these genes, suggesting methylation as an early molecular event in carcinogenesis [31, 34]. However, there is no direct evidence indicating that the level of DNA methylation correlates with recurrence or infiltrative growth of HNSCCs.

In addition to genetic and epigenetic changes, microRNAs (miRNAs) are also involved in the initiation and progression of HNSCCs. MiRNAs are short, single-stranded, 18–23 nucleotide-long, non-coding RNA molecules, which repress the expression of its target genes post-transcriptionally [35]. The mechanisms of miRNA biogenesis and function were described in detail previously [36]. Altered expression of miRNAs may be caused by transcriptional dysregulation of miRNAs, chromosomal aberration, epigenetic changes, single nucleotide polymorphisms (SNPs), or defects in the miRNAs processing machinery, and is closely associated with the initiation and progression of malignancies [37]. Approximately fifty miRNAs have been linked with HNSCC. A full list of HNSCC miRNAs can be found in the database HeNeCan miRs, and is available at http://tarmir.rgcb.res.in/henecan/ [38]. Some HNSCC-related miRNAs, known as oncomirs, possess oncogenic capacity by several mechanisms, such as dysregulating key cell cycle checkpoints to promote unchecked proliferation (e.g., miR-106b) [39, 40], interrupting the crosstalk between tumor growth factor-beta (TGF-β) and Myc pathways (e.g., miR-106b-25/miR-17-92 cluster), which leads to cell cycle dysregulation and resistance to apoptosis [41], or interrupting apoptosis through the PI3K/PTEN/AKT pathway (e.g., miR-21) [40].

HNSCC Initiates with Limitless Proliferation of Epithelial Cells

A cancer cell exhibits uncontrolled proliferation, and invades the surrounding tissue, suggesting that the underlying pathobiological mechanisms involve dysregulation of proliferative signaling pathways, inhibition of growth suppressors, resistance to cell death, and maintenance of replicative immortality [29].

Dysregulation of Proliferative Signaling Pathways

Growth factors stimulate cell proliferation via binding to the surface receptors of target cells, activating kinases with transduction into the nuclei to further activate the intracellular signaling cascade. Epidermal growth factor (EGF) is critical for the initiation and progression of HNSCC. In addition to growth factor ligand-dependent pathways, activation of downstream signaling pathways (e.g., phosphatidylinositol-3-kinase, PI3K/AKT pathway) alone may also lead to the dysregulation of cell proliferation.

EGF receptor (EGFR), a member of the ErbB/Her family, is a transmembrane receptor tyrosine kinase [42]. Once bound with the ligand (e.g., EGF and TGFα), EGFRs activate several downstream signaling cascades, including Ras/Raf/mitogen-activated protein kinase (MAPK), PI3K/AKT, mammalian target of rapamycin (mTOR), Janus kinase (Jak)/signal transducer and activator of transcription (STAT), and protein kinase C (PKC) pathways [43]. The EGFR pathway regulates cell proliferation and survival, and is associated with tumor invasion, metastasis, and angiogenesis [44]. Aberrant activation of EGFR pathway is prevalent in HNSCC [45, 46], which may present as amplification of EGFR, mutation of EGFR, or overproduction of ligands [47]. EGFR variant III (EGFRvIII), a truncation mutated variant, leads to conformational activation of the EGFR without ligand binding or receptor overproduction [48]. Cells with EGFRvIII demonstrated higher resistance to chemotherapy [48].

A monoclonal antibody to EGFR, cetuximab, is the first molecularly targeted therapy to receive US Food and Drug Administration (FDA) approval for treating HNSCC [49]. Cetuximab improved overall survival when used to manage locally or regionally advanced and recurrent/metastatic HNSCCs [50, 51]. Based on the National Comprehensive Cancer Network (NCCN) guidelines, cetuximab plus 5-fluorouracil and platinum (cisplatin or carboplatin) is a category 1 treatment option for patients with unresectable or recurrent/metastatic non-nasopharyngeal HNSCC [52]. Other targeted therapies include tyrosine kinase inhibitors (e.g., lapatinib, dacomitinib, and afatinib) and mTOR inhibitors (e.g., temsirolimus and everolimus) which are currently being used in clinical trials for HNSCC [49].

Inhibition of Growth Suppressors and Resistance of Cell Death

Cancer cells gain limitless replicative potential through altering the cell cycle, within which Retinoblastoma (RB) and TP53, as tumor suppressor genes, play important roles (as will be noted in HPV-related lesions below) [53]. There are two critical check points of the cell cycle in the G1 and G2 phases that are regulated by complexes of cyclins and cyclin-dependent kinases (CDKs). RB protein, a tumor suppressor and a critical regulator in G_1/S cell cycle progression, typically binds to and inactivates transcription factor E2F and the ensuing signaling cascade. When RB is phosphorylated, E2F is released to activate the transcription of c-Myc, cyclin A, and p21/WAF-1, so that cell cycle proceeds [54]. Loss of function

or expression of RB results in uncontrolled cell proliferation. TP53 is a tumor suppressor gene, playing a critical role in cell cycle progression, cell differentiation and apoptosis. TP53 mutation commonly occurs at a hot spot region at codon 245 and codon 248 [55]. Somatic mutations of TP53 were found in 50 %~80 % of HNSCC cases [56–58], and disruptive mutation of TP53 is strongly associated with decreased overall survival rate [58].

Microenvironment Enhances the Progression and Invasion of HNSCC

In addition to the genetic/epigenetic dysregulation within cancer cells, recent studies suggest that the connective tissue may significantly contribute to the progression and invasion of HNSCC [59]. The natural boundary of an epithelium is maintained by normal interactions with the underlying connective tissue and basement membrane [60]. When neoplastic processes begin, the microenvironment demonstrates increased microvasculature, modified extracellular matrix (ECM) deposited by cancer-associated fibroblasts (CAFs), and infiltration of inflammatory cells. The key findings of HNSCC microenvironment are summarized below.

Induction of Angiogenesis

The rapid growth of solid tumors, such as HNSCC, results in hypoxia of the tissue, and induces angiogenesis [61]. Hypoxia activates the secretion of vascular endothelial growth factors (VEGFs), mediated by hypoxia-inducible transcription factor 1α (HIF-1α) and 2α (HIF-2α) within tumor cells [62, 63]. In addition to hypoxia, growth factors (e.g., EGF) or their receptors (e.g., EGFR), cytokines (e.g., IL-6), and protein products of activated oncogenes or mutated tumor suppressor genes, may also regulate the expression level of VEGF [63, 64]. VEGF-A, one of the VEGF family members, is a 45-kDa homodimeric glycoprotein with different isoforms and diverse range of angiogenic activities [65]. VEGF-A is particularly critical in tumor angiogenesis, and has been found overexpressed in both HNSCC cell lines and specimens [66–68]. The overexpression of VEGF-A is correlated with poor prognosis and lymph node metastasis in HNSCC [68–70]. Cancer cells as well as surrounding stromal cells, such as fibroblasts, produce VEGF and encourage neovasculaturization through the VEGF/VEGF receptor (VEGFR) axis, especially VEGFR2, which triggers multiple downstream signaling pathways, leading to the survival, migration, and differentiation of endothelial cells [63]. VEGFR2-mediated survival, proliferation, and migration of endothelial cells are further enhanced by activating pathways of PI3K, MAPK, and focal adhesion kinase, respectively [71, 72]. In addition, VEGF also enhances microvasculature permeability [73]. Therefore, it may provide a path for metastatic dissemination of malignant cells. The calcium-dependent pathway, Akt signaling pathway and Erk1/2 pathway increase the level of nitric acid, cGMP and prostaglandins, also suggesting they play a role in increasing vessel permeability [74, 75]. However, the detailed regulatory mechanisms need further elucidation.

VEGF overexpression has been shown as a critical component regulating angiogenesis in HPV16-positive cervical SCC [76]. E6 viral oncoprotein binds to the promoter region of VEGF and activates the expression level of VEGF [77]. Moreover, in HPV16-positive tumor cells, VEGF can enhance cell proliferative activity through up-regulating EGFR, while in the HPV16-negative cervical cancer, up-regulation of mutated EGFR plays a major role of driving cell proliferation [78]. However, the relationship between HPV16 infection and VEGF expression remains controversial in oropharyngeal SCC [79, 80]. In conventional HNSCC, up-regulation of VEGF is correlated with poor clinical outcome, but this correlation becomes weaker in HPV16-positive HNSCC [80]. Further research on angiogenesis of HPV16-positive and negative HNSCC is required to elucidate the underlying mechanisms.

Interplay Between Cancer Cells and Stromal Cells

In addition to the genetic/epigenetic dysregulation within cancer cells, stromal cells, also known as CAFs (cancer-associated fibroblasts), may be critical in the progression and invasion of HNSCC. CAFs demonstrate myofibroblastic features with cytoplasmic accumulation of α-smooth muscle actin (α-SMA), and have a role in synthesizing ECM, such as type I collagen [81]. In HNSCC, CAFs are frequently associated with dense collagen deposition and stromal desmoplasia [82]. Stromal α-SMA positivity was observed in 100 % of the conventional SCC, but not in the non-neoplastic or adjacent uninvolved stroma. Together with loss of CD34, gain of stromal α-SMA positivity links myofibroblasts and tumor microenvironment with tumor invasion [83]. Up-regulation of α-SMA and integrin-α6 in CAFs is shown to be correlated with invasiveness and poor prognosis of oral SCC [84]. Integrin-α6 is critical for cell-cell adhesion and cell-ECM interaction as well as downstream signaling cascades that regulate cell cycle.

CAFs also produce variable cytokines, such as CXCL12 and matrix metalloproteinases (MMPs), which promote cell motility and invasiveness of HNSCC. The CXCL12/CXCR4 axis, first discovered in the trafficking of hematopoietic stem cells to the bone marrow, plays a key role in modulating tumor microenvironment, as well as up-regulating expression of MMP9 and HIF-1α [85, 86]. CXCL12, also called SDF-1 (stromal cell-derived factor-1), is one of the chemokines that binds to a transmembrane G-protein coupled receptor, CXCR4 [85].

CAFs-derived CXCL12 induces invasion and dispersing of CXCR4-overexpressed oral SCC cells, and is strongly associated with regional lymph node metastasis of oral SCC [87–89]. MMPs (e.g., MMP-2 and MMP-9), a group of proteolytic enzymes that degrade and remodel ECM components, are regulated by multiple signaling pathways (such as EGFR), which are commonly overexpressed in HNSCC [90]. Up-regulation of MMPs in HNSCC is associated with increased metastatic rate and poor prognosis [91, 92]; therefore, the underlying mechanisms are worthy of further investigation.

Epithelial-Mesenchymal Transition Contributes to the Invasion and Metastasis of HNSCC

Epithelial-mesenchymal transition (EMT) is a fundamental biological process in embryonic morphogenesis that plays a critical role in cancer development and progression, and is considered a crucial event linked to tumor invasion and metastasis. The classical EMT features in cancer cells include loss of cell polarity and E-cadherin, as well as acquisition of mesenchymal features, namely spindle morphology, motile phenotype, and expression of mesenchymal markers (e.g., vimentin and SMA) [93].

TGF- β pathway is a major player triggering EMT in HNSCC [94]. In TGF-β signal transduction, dimerized ligands bind to type I and type II receptor heterodimers to phosphorylate and activate the transcription factor Smad. Smad proteins consist of three functional classes, namely the receptor-regulated Smad (R-Smad), the co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad) [95]. R-Smad (Smad1, Smad2, Smad3, Smad5 and Smad8) is directly phosphorylated by type I receptor kinases. TGF-β signaling pathway selectively activates Smad2/3 [95]. The dimerized phosphorylated R-Smads then form a regulatory complex with the Co-Smad (Smad4), and translocate to the nucleus to activate the expression of downstream genes. On the other hand, the I-Smads (Smad6 and Smad7) compete with R-Smads for interaction with the Co-Smad and type I receptor [95].

TGF-β pathway also contributes to EMT through STAT3-mediated activation of Twist, Snail, and Slug [96, 97]. Twist induces Bmi-1, and the two act cooperatively to down-regulate E-cadherin but up-regulate N-cadherin in HNSCC, which is referred to as a "cadherin switching" phenomenon [98]. Slug also contributes to cadherin switching in response of hypoxia-related HIF-1α expression, and is correlated with poor prognosis in HNSCC [99]. Emerging evidence suggests that EMT is a process of gaining "stemness" or becoming CSCs, which may contribute to the resistance to conventional therapies [17, 100].

Emerging Technologies in HNSCC Research

Massively parallel sequencing, also known as the next-generation sequencing, is a high-throughput sequencing technique to analyze the genomes of HNSCC thoroughly and efficiently. Two groups published their results back-to-back in *Science* in 2011, performing exome sequencing for both tumor DNA and the corresponding normal DNA from the same patient [101, 102]. In addition to previously described mutations in HNSCC (e.g., TP53, CDKN2A and PIK3CA), a novel mutation, NOTCH, is identified [103].

There are four members in the mammalian NOTCH receptor family, NOTCH 1–4. When the NOTCH ligand binds to the NOTCH receptor, the intracellular domain translocates into the nucleus and activates downstream genes, such as NOTCH receptors, NOTCH ligands, cyclin D1 (CCND1), and MYC, which are critical for the cell cycle regulation [103]. In addition to TP53, NOTCH mutation is the second most common mutation in HNSCC, occurring in 10 %–15 % of the HNSCC cases [104]. In HNSCC, approximately 40 % of the mutations in NOTCH1 were truncated gene products, suggesting that NOTCH1 may act as a tumor suppressor gene in HNSCC [101]. This novel approach provides us a comprehensive overview of the gene profiles of HNSCC, and contributes significantly to our understanding in cancer biology.

HPV-Associated HNSCC

HPV-associated HNSCC has been recognized as a clinically and biologically distinctive variant of SCC which occurs predominantly in the oropharynx of younger male patients (median age 56.9 years) [105]. HPV is an epitheliotropic, double-stranded DNA virus, with approximately 150 subtypes identified, of which 120 have been fully sequenced [106, 107]. High risk HPVs, mainly HPV16, are associated with 25.9 % of the HNSCC cases, with the highest prevalence in oropharyngeal SCC (35 %–40 %) followed by laryngeal SCC (24 %) and oral SCC (23.5 %) [108, 109]. Moreover, the prevalence of high risk HPV in oropharyngeal SCC has significantly increased from 35 %–40 % before 2000 to 72.2 % in 2005–2009 [110].

High risk HPVs induce carcinogenesis via two important viral oncoproteins, E6 and E7, which cause dysregulation of the cell cycle and apoptosis [106]. Viral protein E7 binds to RB, and causes reduced expression of RB and overexpression of p16INK4a (a gene product of CDKN2A), leading to proliferation and cell cycle progression [107]. Viral protein E6 inhibits wild type TP53, a major factor for the G2 check point, through binding to and triggering of the ubiquitin-mediated degradation of TP53. This results in a compromised ability of the infected cells to engage cell cycle checkpoints and apoptotic responses. In HPV-associated HNSCC, mutated p53 is

not expected in the tumor cells as compared with conventional HNSCC [111]. The molecular consequences of expressing E6 and E7 include interruption of p53-mediated apoptosis and unlimited proliferation. It may be due to the absence of p53 mutation that HPV-associated HNSCC demonstrates better prognosis compared to the HPV-negative HNSCC [112].

Conclusion

HNSCC is one of the leading cancers in the world associated with a high mortality rate. Multiple risk factors have been identified, including tobacco, alcohol, and areca nut consumption, as well as high risk HPV infection. Currently, surgical removal with adjuvant chemotherapy and radiotherapy is still the mainstay of treatment. Emerging technologies contribute to an understanding of the underlying pathobiological mechanisms and help in the development of personalized targeted therapy.

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Compliance with Ethics Guidelines

Conflict of Interest Dr. Chia-Cheng Li and Dr. Sook-Bin Woo each declare no potential conflicts of interest.

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