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REVIEW ARTICLE

Unraveling the molecular genetics of head and neck cancer through genome-wide approaches

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Abstract The past decade has seen an unprecedented increase in our understanding of the biology and etiology of head and neck squamous cell carcinomas (HNSCC). Genome-wide sequencing projects have identified a number of recurrently mutated genes, including unexpected alterations in the NOTCH pathway and chromatin related genes. Gene-expression profiling has identified 4 distinct genetic subtypes which show some parallels to lung squamous cell carcinoma biology. The identification of the human papilloma virus as one causative agent in a subset of oropharyngeal cancers and their association with a favorable prognosis has opened up avenues for new therapeutic strategies. The expanding knowledge of the underlying molecular abnormalities in this once very poorly understood cancer should allow for increasingly rational clinical trial design and improved patient outcomes.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world, with an annual world-wide incidence of over 600,000 cases per year and 350,000 deaths per year.[1] Cancers in the head and neck can originate from a variety of subsites including the lip, oral cavity, nasopharynx, oropharynx, and larynx. The most common causes of head and neck cancers are tobacco and alcohol, which work synergistically, and are responsible for 70–75% of cases.[2,3] In parts of Asia, betel-quid chewing also plays a significant role in the development of malignancy.[4] On a molecular level, these cancers often have p53 mutations and many display chromosomal instability.[5]

More recently, the human papilloma virus (HPV) has been shown to promote HNSCC and primarily cause oropharyngeal cancers (a sub-site in the head and neck).[6] Researchers have found significant differences in the pathogenesis and prognosis of HPV-related malignancies and HPV-negative

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malignancies and have tended to classify HPV associated malignances as a separate biologic entity. Unlike HPV-negative cancers, HPV-related oropharyngeal malignancies are not as strongly associated with tobacco and alcohol exposure. They are, instead, related to sexual behavior, which is a conduit to transmit HPV.[7,8] Relative to HPV-negative malignancies, HPV-positive cancers are associated with a more favorable prognosis.[7,9] The incidence of HPV-positive cancer is rising, while incidence of HPV-negative cancers is decreasing.

Treatment of HNSCC is made challenging due to the diversity of the anatomic sites in the head and neck and the critical normal structures that may be near a particular tumor site. Often, the care of a patient requires a multidisciplinary team of surgeons, radiation oncologists, medical oncologists, nutritionists, gastroenterologists, and speech and swallowing therapists, amongst others. Treatment options for HNSCC can consist of definitive surgical resection, definitive chemo-radiation, or a combination of surgery and chemo-radiation. Despite the availability of aggressive treatments, the 5-year survival rate for head and neck malignancies remains relatively poor at 65%, with only modest gains in the past few years.[10]

An understanding of the molecular and genetic abnormalities leading to oncogenesis in head and neck malignancies has dramatically increased in the past decade. Initial attempts to understand the genetic etiology of head and neck cancers focused on cytogenetic studies. The development of microarray technology enabled the classification of HNSCC into distinct types based on gene expression. More recently, next-generation sequencing technologies have allowed multiple groups of researchers to sequence a large number of tumors to identify novel mutated tumor suppressor genes and oncogenes. An underlying rationale for these researchers' genomic profiling studies was to gain a more thorough understanding of the molecular abnormalities in head and neck cancer to help guide the development of new therapeutics. Before reviewing the major discoveries of each of these profiling techniques, we will briefly discuss genetic factors that may pre-dispose individuals to develop HNSCC.

Genetic predisposition to HNSCC

Although most cases of HNSCC are induced by carcinogens or viral infection, a small fraction of cases are familial in nature. The syndromic etiology with the clearest link of an increased risk of HNSCC is Fanconi anemia, an autosomal recessive genomic-instability syndrome associated with bone marrow failure, leukemia, congenital defects, and sensitivity to cross-linking agents.[11] Cohort studies of Fanconi anemia patients have demonstrated a highly elevated risk of HNSCC with the ratio of the observed number of HNSCC cases to expected number of cases ranging between 240 and 706.[12–14] Treatment of these patients requires care as they are sensitive to common chemotherapeutics used in HNSCC and may be sensitive to radiotherapy as well, although this latter point is controversial.[15] Rare clusters of HNSCC have also been reported in families with germ-line mutations in *CDKN2A* and *ATR*.[16,17]

In non-syndromic families, initial case–control studies demonstrated a genetic predisposition, with first-degree relatives having a 3.5 fold increased risk of HNSCC. Subsequent pooled analysis, however, led to an odds ratio of 1.7.[18–21] It has been hypothesized that genetic differences in pathways such as DNA repair, carcinogen metabolism, and cell cycle control may increase the risk of carcinogenesis from exposure to tobacco or alcohol.[22] Phenotypic differences in these processes in lymphocytes from HNSCC patients and controls have been identified.[23,24] Candidate gene-based studies have generated mixed results, with the notable exception of a nearly 9000 patient series that identified SNPs in multiple alcohol dehydrogenase (ADH) genes associated with a decreased risk of an upper aero-digestive malignancy.[25–28] Subsequently, a genome-wide association study in upper aero-digestive malignancies validated the ADH SNPs and also identified a SNP in *HELQ* (a DNA repair gene) as being associated with risk of malignancy. Cumulatively, these SNPs only accounted for 4% of the familial risk.[29]

Expression profiling

One of the first attempts to genomically profile HNSCC was with mRNA profiling. Unsupervised hierarchal clustering of the transcriptome of 60 HNSCC patients[30] identified 4 subtypes — basal, atypical, mesenchymal type, and classical subtype — which have subsequently been verified by other investigators.[31] Classical tumors exhibit alterations in expression of genes involved in oxidative stress, such as KEAP/NFEL2. The atypical cluster is enriched with HPV-positive tumors (discussed further below). Mesenchymal tumors demonstrate an elevation in expression of genes associated with epithelial-to-mesenchymal transition (EMT). The basal subtype has an expression pattern similar to basal epithelial cells in airways and is named for its similarity to the basal type in squamous cell carcinoma of the lung.

The four subtypes do not show a significant correlation with age or smoking status, but do appear to be related to site of origin.[31] Still, each anatomic subsite, except for hypopharyngeal cancer, is present to some extent in each cluster, suggesting that expression-based subtypes reflect a biology that, at least in part, transcends anatomic sub-site. Whether these subtypes provide prognostic information in and of themselves is unclear at present, given the small size of the studies to date examining the issue. Initial reports indicated these expression-based groups may predict for recurrence-free survival, however, those findings have not been subsequently replicated.[31] Of significant interest is that there is a strong correlation between each of these subtypes and their corresponding expression-based subtypes in squamous cell carcinoma of the lung.[31] That is, the basal, mesenchymal, and classical subtypes in HNSCC strongly correlate with the basal, secretory, and classical subtypes in lung cancer. In other malignancies such as breast cancer and glioblastoma, expression-based subtypes have helped guide translational research as well as therapeutic development; their utility in HNSCC remains to be determined but they potentially could guide research efforts.[32,33]

Table 1 Major sequencing studies.

	Stransky	Agrawal	Pickering	India ICGC
N	74	32	40	50
Disease sites[a]	OC, OP, H, L, SC	OC, OP, L, H	OC	OC
Smokers	89%	75%	78.6%	96%
HPV positivity	14%	25%	2.5%	26%
Mean coverage	150	77[d]	119	38[d]
Whole exome	72	32	40	50
Whole genome	2	0	0	0
Additional tumors with limited sequencing	NA	88	NA	60
Significantly mutated genes[b]	39	6	53	10
Mutations per MB (HPV+; HPV−)	2.28; 4.83	4.8; 20.6	NR[c]	4.07; 3.36

[a] Sites: OC: Oral Cavity, OP: Oropharynx, H: Hypopharynx, L: Larynx; SC sinonasal cavity.
[b] Significantly mutated here is as determined by authors of paper (some studies included formal statistical testing, while others did not).
[c] Not reported.
[d] Group used multiple sequencing platforms, highest mean coverage is reported.

Multiple investigators have developed expression-based signatures to predict clinical behavior of HNSCC, such as lymph node metastasis, hypoxia, or radiosensitivity.[34–38] Roepman developed a 102 gene signature that is predictive of the propensity for lymph node metastasis which theoretically could be used to guide decisions regarding lymph node dissections.[34] The predictor, however, was only able to correctly determine lymph node status in 61 of 82 patients, and the authors noted that this was of only incremental improvement to clinical decision-making. Onken et al developed an expression signature for nodal metastasis from a mouse model of oral cavity cancer.[39] Interestingly, this signature could predict nodal metastasis development for human oral cavity cancers in a training set and a small validation set. However, additional validation is still needed.

Several groups have looked into expression signatures to identify tumors that are hypoxic, and therefore, likely to be resistant to radiotherapy. Some groups have looked for hypoxic gene signatures in head and neck cancers whereas others have performed combined analysis over multiple cancers. In general, these signatures have demonstrated prognostic value in small cohorts.[35–37] The hypoxic signature by the Danish group was used to retrospectively analyze data from the DANHCA 5 trial, which was a randomized trial of radiotherapy ± nimorazole (a hypoxic radiosensitizer) in HNSCC.[40] This group found that the benefit of nimorazole on local control and DFS was limited to patients whose tumors were deemed to be "more hypoxic" by their expression signature.[41] This suggests that their signature may be predictive and could help select patients for future clinical trials using hypoxic sensitizers.

Mutational analysis and alterations in specific genes and pathways in HNSCC

The first two large-scale sequencing efforts in HNSCC identified a number of recurrently mutated genes. The majority of these were in tumor suppressor genes rather than oncogenes.[42,43] Stransky et al performed whole exome-sequencing on 74 tumor/normal pairs, with 150-fold mean coverage, while Agrawal et al sequenced 32 tumor/normal pairs, with a mean coverage of 77-fold and performed targeted follow-up sequencing in an additional 88 patients. For HPV-negative tumors, Stransky et al found a mutation rate of 4.83 per Mb while Agrawal et al found a mutation rate of 20.6 per Mb. The large discrepancy between the two groups may have arisen from differences in bioinformatics techniques of mutation calling or differences in coverage of sequencing. Since these initial studies, two other groups have looked at mutations specifically in oral cavity cancers[44,45] (Table 1 for study details).

In a pan-cancer analysis, Stratton and colleagues identified HNSCC as possessing the 9th highest average mutational load out of 30 tumor types studied, with patients having a range of mutations from less than 1 mutation per Mb to over 100 mutations per Mb.[46] They identified several active mutational processes in HNSCC, including mutations due to tobacco exposure, aging, UV light, and alterations in the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) enzymes. Multiple groups have found signatures of tobacco exposure in HNSCC, which

Table 2 Frequency of selected genes recurrently mutated in 4 major sequencing studies to date.

Gene	Stransky	Agrawal[a]	Pickering	India ICGC
p53	62%	47%	66%	62%
CDKN2A	12%	9.2%	0%	2%
CASP8	8%	3%	10%	34%
FAT1	12%	0%	28%	40%
NOTCH1	14%	15%	9%	16%
HRAS	5%	4%	9%	12%
PIK3CA	8%	6%	11%	6%
MLL2	11%	0%	9.5%	10%
FBXW7	5.4%	5%	4.7%	2%

Red indicates genes were not identified as significant in that study.
[a] Did not use formal statistical testing.

often include guanosine-to-thymidine transversions.[43,46] Although alterations in APOBEC have been shown to cause mutations in other malignancies, most prominently breast cancer, it is not clear these enzymes are active in HNSCC as of yet.[47] Instead of looking at the type of mutations, Mroz et al used sequencing data to estimate tumor heterogeneity, which they called the mutant-allele tumor heterogeneity (MATH) score. This score was prognostic in both HPV-positive and HPV-negative patients.[48]

In HPV-negative tumors, the predominant finding from these sequencing efforts was that a large number of tumor suppressor genes were mutated. The Broad group identified 39 genes with recurrent mutations ($q \leq 0.1$), whereas the JHU group identified 6 genes with mutations in more than 4% of their study (Table 2). Mutations and other genomic alterations in individual genes and key pathways are reviewed individually below. Alterations in selected pathways are summarized in Fig. 1. Of the non-coding mutations, an interesting one occurs in the promoter of *TERT* in nearly 20% of HNSCC, resulting in increased expression of telomerase.[49]

TP53

Long before the era of genome sequencing, TP53 had been recognized as a frequently mutated gene in HNSCC, with modern series estimating 46–73% of HNSCC cases having a mutation.[42–44,50–53] TP53 is a tumor suppressor gene that functions primarily as a transcription factor and regulates the expression of hundreds of downstream target genes in response to a variety of cellular stresses.[54,55] It is composed of 393 amino acids and contains 4 domains, including a highly conserved DNA binding domain (DBD). It plays a key role in a number critical cellular functions, including the response to DNA damage and oncogenic stress. DNA damage leads to activation of p53, triggering cell cycle arrest and an attempt to repair the damage, which if not completed, can trigger apoptosis or senescence. Mutations in TP53 appear to be acquired early in the pathogenesis of HNSCC and are present in pre-malignant lesions, suggesting an important role in early oncogenesis.[5,51,56]

Most of the missense mutations in TP53 cluster in the DNA binding domain (DBD) whereas frame-shift and nonsense mutations are more equally distributed throughout the gene. One third of the mutations in the DBD occurs within 6 hotspot residues and result in preventing binding to DNA.[55] Since p53 forms a tetramer, many of these missense mutations are expected to have a dominant negative effect.[57] Work has indicated that some of these mutations may also result in a "gain-of-function" phenotype, perhaps resulting from expression of other off-target genes. However, the significance of this remains unclear.[57,58] Besides mutation, p53 may be inactivated in other ways such as via *MDM2* over-expression or amplification (MDM2 is a negative regulator of p53) or deletion of *CDKN2A* (negatively regulates MDM2).[59] Inactivation of p53 also occurs in HPV-positive tumors and is discussed in more detail below.

Whether alterations in TP53 are prognostic for overall survival has been somewhat controversial, with early studies showing conflicting results.[54,60] Limitations in these earlier studies include the reliance on immunostaining,

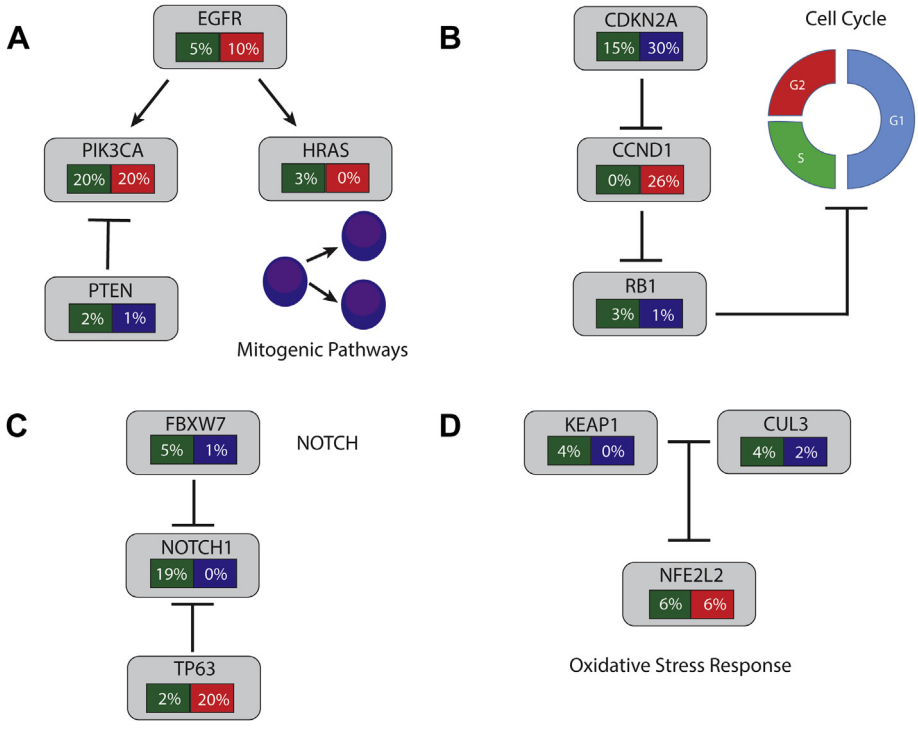

Figure 1 Alterations in selected pathways in head and neck squamous cell cancers. Green: Frequency of mutations. Red: frequency of amplification. Blue: Frequency of deletion. (A) Mitogenic Pathway Alterations. (B) Cell Cycle Alterations. (C) NOTCH Signaling. (D) Oxidative Stress Response. See text for more details of alterations in each pathway (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

which is often discordant with mutational status, and a failure to sequence the entire length of the TP53 gene.[61,62] More recent and thorough sequencing-based analysis shows that functional mutations appear to result in significantly worse outcomes.[50,63] For instance, Poeta and colleagues examined a cohort of 420 patients treated with surgery ± radiotherapy and found that mutations in TP53 were associated with worse overall survival (HR = 1.4, $p = 0.009$). Patients with truncating mutations or disrupting mutations in the DNA binding domain had significantly worse overall survival (HR = 1.7, $p < 0.001$), whereas non-functional mutations alone were not statistically associated with worse outcomes (HR = 1.2, $p = 0.16$).

As p53 plays an integral role in handling genotoxic stress, multiple groups found that TP53 mutation results in worse outcomes in patients uniformly treated with either definitive radiotherapy or post-operative radiotherapy.[63–65] However, as TP53 mutations also are prognostic in patients treated with surgery alone, it is unclear if mutational status is a true predictive marker. Further, the mechanism by which *p53* promotes radio-resistance remains unclear.[66] In cell types susceptible to p53-mediated apoptosis, loss-of-function would theoretically induce radio-resistance. *p53*-mediated apoptosis, however, does not appear to be the dominant mode of cell death in epithelial tumors.[66] Recent *in-vitro* work with HNSCC cell lines has suggested that a subset of *p53* mutations may have a gain-of-function phenotype that promotes senescence and subsequent radio-resistance.[65] Future investigation and more accurate models will be needed to sort out this controversy.

Cell cycle alterations: CDKN2A, CCND1, and RB1

There is homozygous deletion of CDKN2A in nearly 30% of HNSCC and the gene is mutated in another 10–20% of samples, and is frequently subject to LOH.[67–69] The majority of mutations are inactivating and includes frame shifts, nonsense mutations, and splice site changes. Additional epigenetic alterations may lead to this gene being inactivated in up to 80% of tumors.[70] One possible strategy to target this alteration would be to inhibit CDK4/CDK6. Recently, Palbociclib, a CDK4/6 inhibitor, increased progression free survival in a cohort of breast cancer patients, suggesting such an approach may be efficacious.[71]

The next most commonly altered cell cycle gene in HNSCC is *CCND1*, which is amplified in over 20% of tumors.[68] *CCND1* encodes cyclin D1, which serves as a cofactor for CDK4 and CDK6 to phosphorylate Rb.[72] Again, CDK4/6 inhibitor could serve as a method to target these tumors, and both p16 loss and *CCND1* amplification are being investigated as biomarkers in the Palbociclib trial. In the TCGA data, genetic alteration of one of either *CCND1* or *CDKN2A* occurs in nearly 60% of cases and in the study by Pickering et al, it was 94%.[45] *RB1*, the retinoblastoma gene, also appears to be either mutated or deleted in approximately 5% of patients.

EGFR

Epidermal growth factor receptor (EGFR) is a member of the HER/erbB family of receptor tyrosine-kinases and is one of the primary targetable alterations in cancers.[73,74] When activated by binding its ligand, EGFR homodimerizes or heterodimerizes with other members of the ErbB family, stimulating its tyrosine kinase activity and leading to autophosphorylation of tyrosine residues in its cytoplasmic tail.[75] This can lead to activation of downstream signaling cascades including RAS/RAF/MAPK, PI3K/AKT/mTOR (discussed further below), and/or JAK/STAT. These pathways are important regulators of proliferation, invasion, angiogenesis and metastasis.

Therapeutics targeting the EGFR pathway have been introduced into the clinical setting for a number of malignancies, including HNSCC.[76,77] Cetuximab, a chimeric murine/human monoclonal antibody against EGFR, has improved overall survival compared to standard treatment in the locally advanced and metastatic settings, although adoption in the locally advanced setting remains controversial.[78–81] Two humanized antibodies against EGFR, however, have failed to improve overall survival in either the recurrent/metastatic setting or the locally advanced setting.[82–84] Similarly two first-generation, small molecule, tyrosine kinase inhibitors against EGFR (gefitinib and erlotinib) have been disappointing.[85–87]

Although EGFR is expressed on the surface of the majority of HNSCC[88] tumors and the degree of overexpression is associated with a worse prognosis, the muddled picture from therapeutic interventions in the pathway has led to uncertainty on exactly how this pathway is activated in HNSCC.[89–92] Unlike adenocarcinoma of the lung where mutations in the kinase domain result in constitutive activation of EGFR, such mutations are rare in HNSCC.[69,93] Amplification of EGFR occurs in a minority of patients and the presence of amplification or increased IHC expression has not correlated with efficacy of EGFR inhibition.[94,95] Of note, amplifications in EGFR appear to occur less frequently in HPV-positive patients, although the therapeutic implications of this are unclear.

The molecular and clinical trial results suggest that perhaps cetuximab's efficacy is mediated more by its antibody-dependent cell-mediated cytotoxicity then blockade of the EGFR pathway.[76,96,97] Alternatively, resistance to EGFR inhibition may occur quickly by up-regulation of other erbB receptors, such as ERBB2 (HER2/NEU).[98] Strategies addressing both these possibilities are being actively pursued clinically with the development of pan-erbB inhibitors and monoclonal antibodies as well as the pairing of cetuximab with various immune check-point inhibitors in development.

PI3K/AKT/mTOR

PIK3CA is the 2nd most commonly mutated gene across human cancers and alterations in this pathway are thought to play an important role in cancer cell growth, survival, and metabolism.[99,100] The PI3K family of kinases consists of three classes, with class IA being most frequently involved in cancer. Activation of PI3K leads to phosphorylation of phosphatidylinositols (PIP_2, a second messenger) and subsequent activation of AKT, one of the main effectors of PI3K signaling.[100] Activated forms of AKT have been found in the majority of HNSCC tumors analyzed by tissue microarrays.[88]

AKT further activates mTOR, which is also responsible for integrating cellular signals regarding nutrient levels, cellular energy stores, and stress. One mechanism of PI3K activation in HNSCC is by receptor-tyrosine kinases, such as EGFR. Although EGFR cannot directly active PI3K, if EGFR heterodimerizes with ERBB3 it can subsequently activate PI3K signaling.[101]

Another mechanism of activation of PI3K pathway in HNSCC involves mutation, with mutations occurring in PI3K catalytic subunit, p110α (encoded by *PIK3CA* gene). Approximately 20% of tumors have mutations in PIK3CA, however these mutations occur much more commonly in HPV-positive tumors compared to HPV-negative tumors.[68] Mutations often occur in one of two hotspot locations in the kinase or helical domain and promote constitutive signaling through the pathway.[102] Further, *PIK3CA* is amplified in 20% of HNSCC overall. PTEN loss or mutation occurs in 3% of HNSCC. PTEN encodes a phosphatase that shuts off PI3K signaling by dephosphorylating PIP_3 to PIP_2. Genomic alterations in other members of the PI3K pathway typically occur at frequencies of less than 5% in HNSCC.

Multiple therapeutic agents that target this pathway are under investigation in both the upfront and metastatic/recurrent setting. In general, agents targeting this pathway include sole PI3K inhibitors (isoform selective and pan-inhibitors), dual PI3K-mTOR inhibitors, AKT inhibitors, and mTOR inhibitors. In other malignancies, responses to these drugs as single agents have been disappointing and perhaps suggest the development of feedback loops counteracting inhibition.[103] Further, initial experience with dual PI3K-mTOR agents appear to be limited by toxicity. To-date, however, experience in HNSCC remains limited and trials are presently on-going.[77]

NOTCH1/p63/FBXW7

The discovery of recurrent mutations in *NOTCH1* was one of the key novel findings of the initial HNSCC exome-sequencing projects.[42,43] Notch1 is a transmembrane receptor that plays a role in cell differentiation and embryonic development.[104,105] Signaling is thought to be mediated by cell-to-cell contact, enabling binding of Notch to one of five known ligands. After receptor activation by ligand binding, a cascade of proteolytic cleavages take place that result in the release of Notch1's intracellular domain (NICD). NICD subsequently translocates to the nucleus where it interacts with CBF1 (CSL) and mastermind-like (MAML1) leading to transcriptional activation of multiple genes. Well characterized genes under control of this pathway include the HES and HRT family of genes, notch ligands, *CCND1*, *MYC*, and others.

Notch appears to be able to function as either a tumor suppressor or an oncogene depending on cell context. In TCGA data, *NOTCH1* is mutated in 18.6% of cases, with over 35% of mutations leading to a frameshift or nonsense codon.[68] The majority of missense mutations appear in EGF-like domain repeats, which are important for ligand binding. This pattern of mutations has suggested that Notch's role in HNSCC may be as a tumor suppressor gene, although the exact mechanism of tumor suppression remains unclear. In a murine model, deletion of *Notch1* results in hyperplasia and carcinogen-induced skin cancer, supporting a tumor suppressor function.[106] Similar loss-of-function mutations have been seen in cutaneous SCC and lung SCC and cell-based testing of mutations in the EGF-like domain have demonstrated loss of signaling.[107] In contrast, Notch1 mutations are oncogenic in T-cell acute lymphoblastic leukemia and cause ligand-independent cleavage of Notch1, ultimately leading to constitutive activation.[108] Notch's oncogenic functions are thought to be mediated by prompting cell cycle progression and inhibition of apoptosis.[105]

The other Notch receptors are mutated in 2–4% of cases with a roughly similar mutational spectrum. In keratinocytes of both human and mouse origin, Notch1 signaling interacts in a negative feedback loop with TP63. TP63 is a p53 family member gene, which has a role in keratinocyte cell fate determination. TP63 is found amplified in 20% of tumors in the TCGA set and interestingly, amplifications appear to be mutually exclusive with *NOTCH1* mutation ($p = 0.053$).[68] FBXW7 is ubiquitin ligase that targets Notch for degradation and is found mutated in 4.7% of cancers of HNSCC.[109] FBXW7 may have a role outside of Notch signaling, as it targets many other known oncogenes including cyclin E, MYC, and JUN.

Oxidative stress response: NRF2/KEAP1/CUL3

Genes involved in oxidative stress are altered by mutation or copy number change in nearly 20% of HNSCC, and by IHC, NRF2 is overexpressed in 90% of tumors.[68,110] NRF2 (encoded by the *NFE2L2* locus) is a transcription factor that activates the cellular antioxidant response.[111] It is typically constitutively expressed under basal conditions and kept in the cytoplasm and degraded by KEAP1 and CUL3. CUL3 is a core component of an E3 ubiquitin ligase complex, which relies on KEAP1, a substrate specific adaptor, to successfully ubiquinate NRF2 and promote its degradation. The interaction between NRF2 and KEAP1 is mediated by NRF2's Neh2 domain and KEAP1's Kelch/DGR domain. In the presence of oxidative stress, the interaction between KEAP1 and NRF2 is inhibited, leading to the accumulation of NRF2 in the cytoplasm, subsequent translocation to the nucleus, and thereafter transcriptional activation of its target. Increased activation of NRF2 may promote cancer cell growth and protect against cytotoxic therapies.[111]

In HNSCC, *NRF2* is mutated in 6% of patients and amplified in another 6%. Mutations are clustered in the Neh2 domain, specifically in two motifs important for binding with Keap1. The mutations appear to be gain-of-function mutations which inhibit interaction with *KEAP1*, and subsequently lead to an up-regulation of *NRF2*.[112] *KEAP1* is mutated in 4% of tumors and rarely homozygously deleted. The majority of mutations are missense mutations and they occur both in domains involved with binding to NRF2 and those responsible for binding to *CUL3*. These also presumably lead to increased cytoplasmic NRF2, and subsequent activation of the pathway. Lastly, *CUL3* is mutated or deleted in approximately 6% of patients.

In the TCGA data set, genomic alterations in the NRF2 pathway have correlated with worse overall survival.[113] Although not yet validated in another HNSCC cohort, alterations in the NRF2 pathway may be prognostic in other

malignancies such as NSCLC.[114] One reason for worse outcomes would be resistance to cytotoxic treatments. Multiple studies have shown that elevated NRF2 levels lead to chemoresistance in a variety of cancer cell lines that appears to be reversible with siRNA inhibition of *NRF2*.[111,115,116] Similarly, *NRF2* alterations as determined by expression signature also correlate with resistance to radiotherapy in cell-line models.[117]

Chromatin related genes: MLL2, NSD1, EP300, and other epigenetic changes

The discovery of recurrent mutations in chromatin modifying genes was one of the major discoveries of cancer sequencing projects in general. In HNSCC, several chromatin related genes are recurrently mutated, and at least one of these genes is mutated in 32% of cases. Chromatin consists of proteins and DNA and facilitates the packaging of genomic DNA within the nucleus. The basic functional unit is a nucleosome, which consists of a histone octamer and 147 bp of DNA. Chromatin modifying genes can covalently modify histone proteins, which alters the structure of the nucleosome or alternatively alter DNA.[118] These modifications play a critical role in regulating DNA-based processes, such as transcription and DNA repair.

MLL2 (aka KMTD2) is a histone methyltransferase that is mutated in 19% of HNSCC. Over 50% of the mutations lead to a frame-shift, truncation or splice site alteration, suggesting a tumor suppressor function. MLL2 can methylate several lysine residues on histones, including H3K4, which is often associated with positive gene regulation. In leukemia's, *MLL* translocations are quiet common and their role in carcinogenesis is much better understood than the role of the more recently discovered MLL2 point mutations.[119,120] NSD1 is another histone methyltransferase that is mutated in 10% of patients, with, again, a strong bias towards truncating and nonsense mutations.

EP300 is histone acetyltransferase that is mutated in 7% of HNSCC. EP300 and its paralog CBP (CREB-binding protein) are transcriptional co-activators, which share 75% sequence similarity and although they have some distinct roles, they share many functions.[121] Acetylation of histones by EP300 leads to relaxing the superstructure of chromatin and enables the transcription of proximal genes. EP300 can also directly interact with a wide range of transcription factors, pro-proliferation proteins, and tumor suppressors directly, including c-MYC, c-MYB, CREB, c-JUN, c-FOS, p53, STAT1, BRCA1, and SMAD homologous proteins. Given its cooperation with such a wide variety of proteins, it can promote opposing cellular processes depending on cell type and context.

FAT1/CASP8

Clustering analysis of mutations reported by the International Cancer Genome Consortium (ICGC) demonstrated that FAT1 and CAPS8 mutations may define a distinct subtype of HNSCC.[44] FAT1 is mutated in 23% of tumors, with a predominance of nonsense and frame-shift mutations, suggesting a tumor suppressor function. FAT1 encodes a large transmembrane protein, a protocadherin, which contains 30+ cadherin repeats as well as 4–5 EGF-like domains.[122] FAT1 is thought to suppress cancer cell growth by playing a role in Wnt signaling by binding to β-catenin and limiting its ability to translocate to the nucleus.[123] Truncating mutations would inhibit this functionality and promote tumor cell growth. Of note, the FAT family of genes (FAT1-4) are also mutated in glioblastoma, colorectal cancer, gastric, ovarian, and pancreatic cancers. CASP8 is mutated in 9% of HNSCC and encodes a caspase that plays an important role in programmed cell death (apoptosis). CASP8 is specifically involved in the extrinsic pathway of apoptosis signaling and is activated by membrane bound receptors such as Fas and TNF.[124] Upon activation, Casp-8 triggers the execution phase of apoptosis.

Chromosomal instability and copy number alterations

Among HPV-negative HNSCC, 80% display significant chromosomal instability and over 40% of tumors have undergone whole genome duplication.[5,45,125] The mechanisms underlying this instability are unclear, but include both alterations at the chromosomal arm-level and smaller focal alterations (megabase-pair in size).[126] The amount of chromosomal instability is known to vary between gene-expression defined molecular subtypes.[31] On average, an individual tumor will have 7.3 arm-level alterations and 16 focal alterations.[45] TCGA and others have identified a large number of recurrent alterations, including 12 arm-level amplification events, 14 arm-level deletions, 26 focal amplifications, and 49 focal deletions.[69] Some of the most commonly lost regions include 3p, 4p, and 9p, whereas those commonly gained include 3q, 7p, and 8q.[127,128]

Califano and colleagues studied 87 lesions — including hyperplasia, dysplasia, carcinoma *in situ*, and invasive cancer — using microsatellite analysis to determine which chromosomal losses occurred early in carcinogenesis.[129] These investigators identified losses on 3p and 9p as early events that often occur in premalignant lesions. Chromosome 9p contains several tumor suppressor genes, the most notable being *CDNK2A*, which is involved in cell cycle regulation as discussed previously. The tumor suppressor(s) on 3p responsible for oncogenesis in HNSCC remains unclear.[130] Recent genomic analysis of squamous cell carcinoma of the esophagus also identified common deletions in 3p and 9p, and overall demonstrated a very similar copy number landscape to HNSCC, suggesting a close molecular relationship.[131]

Increasing levels of chromosomal instability in premalignant lesions have been associated with an increased risk of progression to malignant disease in multiple retrospective studies.[132–134] Across a variety of malignancies, once cancer has already developed, increasing chromosomal instability also appears to be associated with worse outcome.[135] Similarly, in head and neck cancer, increasing chromosomal instability has been associated with a worse outcome and a more severe malignant phenotype in multiple studies, including worse overall survival and higher risk of lymph node metastasis.[136–138] In terms of predicting response to treatment, one small case control study compared the copy number profile of tumors sensitive to chemoradiotherapy vs. those that were resistant and found a difference in the patterns of gains and losses.[139]

HPV

Human Papillomavirus (HPV) family of viruses are double-stranded DNA viruses with a propensity to infect squamous epithelium.[140,141] The HPV family is composed of both low and high-risk strains, which is determined by a strain's ability to lead to malignant progression. HPV has long been known to induce malignancies such as cervical, anal, and vulvar cancers. Definitive evidence linking HPV as a causative agent in oropharyngeal cancer didn't emerge until the turn of the century.[6] Since then, it has become clear that HPV-related oropharyngeal cancers are a distinct entity with a better prognosis compared to traditional smoking and alcohol related head and neck cancers.[9] Further, epidemiologic trends have suggested a dramatic increase of HPV-related malignancies over the past two decades, with the primary risk factor relating to sexual behavior.[7,142] HPV16 is the main subtype associated with HNSCC, although a number of other subtypes have also been reported.[7,142,143] The role of HPV in the pathogenesis of non-oropharyngeal head and neck cancers remains unclear at present and is an area of active investigation.

The HPV genome consist of 8000 basepairs and is divided into 3 segments, an early coding region (E), a late coding region (L), and a long control region (LCR).[141] The early and late regions contain genes important for different parts of the viral life cycle whereas the LCR contains regulatory elements important for replication and transcription. In cancer, the HPV genome can exist either in an episomal form or integrated into the host genome. The viral load and transcription of HPV-related genes in tumors can vary on the order of several magnitudes between different tumors.[143,144] Integration into the host genome was thought to favor common fragile sites, but can occur randomly. Recent work has suggested recurrent insertions in RAD51 and ERBB2.[143,145] Integration is also thought to promote oncogenesis.

HPV contains two oncogenes, E6 and E7, that inactivate p53 and Rb respectively and are thought to be important mediators in producing a malignant phenotype. E6 reduces p53 activity by binding to E6-AP (UBE3A), a ubiquitin-protein ligase, and targeting p53 for ubiquitination and degradation.[146] E6 can also promote transcription of hTERT, facilitating immortalization of cells. Inhibition of Rb by E7 leads to cell cycle progression as discussed above (see Cell Cycle alterations). E7 inhibits Rb by facilitating ubiquitination and degradation of the protein. Although the E7 protein from low-risk HPV serotypes is also able to bind to Rb, E7 from high-risk HPV serotypes is much more efficient. Both E6 and E7 also interact with a variety of other host proteins that may also play a role in oncogenesis.[141]

Given their distinct etiology, the mutational, expression, and copy number profiles of HPV-related cancers are distinct from their HPV-negative counterparts. For instance, both of the initial sequencing projects demonstrated 2—4 fold less mutations per megabase for HPV-positive tumors compared to their HPV-negative counterparts.[42,43] The Indian ICGC sequencing project did not identify a difference in mutation rate between HPV-positive and negative tumors. However their samples consisted entirely of oral cavity tumors, where the role of HPV in the underlying pathogenesis of malignancy remains unclear.[44] Similarly, which genes are mutated is strongly influence by whether or not the malignancy is HPV-related. For example, p53 is almost never mutated in HPV-related malignancies, as p53 is inhibited by E6. In addition, mutations in PIK3CA appear to be more common in HPV-positive malignancies compared to HPV-negative malignancies (see above). In terms of expression profiling, HPV-related malignancies cluster with atypical tumors; however, not all atypical tumors are HPV-related.[31]

As HPV-positive tumors have a better prognosis, a number of clinical trials are underway investigating various ways of de-escalating treatment to reduce morbidity of treatment. The molecular differences between HPV-positive and negative tumors clearly play a role in their prognosis and will also help guide de-escalation trial design. For instance, some have found an inverse relationship between HPV and the presence of EGFR by IHC staining.[147—149] However, at present there is no definitive clinical data to suggest a difference in efficacy for anti-EGFR therapy in HPV-positive compared to HPV-negative patients.[150]

Conclusion and future directions

Sequencing efforts and integrative genomics have revealed a great deal about the molecular abnormalities that underlie HNSCC. Unfortunately, they have also demonstrated marked heterogeneity, which will make optimizing therapeutic intervention difficult. In HNSCC, oncogenic mutations are rare and mutations in tumor suppressor genes dominate. Restoring function of a tumor suppressor pharmacologically is difficult. Targeting these alterations may require exploring synthetic lethal approaches.[43] Although any individual tumors may still have some targetable alterations (i.e. amplification or mutation in a known oncogene), it is unclear in many cases if these are passengers or driving events.[45] Furthermore, oncogenic pathways can be activated by non-genetic mechanisms, which would not have been detected by genomic efforts. Now that a genetic blueprint for HNSCC has been completed, the challenge moving forward will be to identify ways to use this information to develop improved diagnostic and therapeutic modalities.

Conflict of interest

The authors have no conflict of interest to disclose.

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