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Oral Cavity and Oropharyngeal Squamous Cell Carcinoma Genomics

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Synopsis

Head and neck squamous cell carcinoma (HNSCC) results from the accumulation of genetic and epigenetic changes in a variety of cellular pathways. The study of tumor development and progression is complicated by the biological complexity of this disease. Recent technological advances now permit the study of the entire cancer genome, which can elucidate complex pathway interactions that are not apparent at the level of single genes. In this review, we describe innovations that have allowed for whole-exome/genome analysis of genetic and epigenetic alterations, as well as of changes in gene expression. Studies using next-generation sequencing, array comparative genomic hybridization, methylation arrays, and gene expression profiling are reviewed, with a particular focus on findings from recent whole-exome sequencing projects. A discussion of the implications of these data on treatment and future goals for the field of cancer genomics is included.

Introduction

Head and neck squamous cell carcinoma (HNSCC) results from the accumulation of multiple genetic and epigenetic changes in a variety of cellular pathways. The processes of genetic alteration and selection result in the clonal expansion of those cells with the most favorable genetic aberrations, resulting in tumor development and eventual progression to invasive carcinoma ^{1,2}.

The development and progression of cancer involves changes within multiple pathways with complex interactions ^{3,4}. The study of the molecular underpinnings of HNSCC is further complicated by the biological complexity of the disease. HNSCC is now known to be heterogeneous at both the histopathologic and molecular levels ^{5–7}. The most prominent distinction is between human papillomavirus (HPV)-positive and HPV-negative tumors, but other subclasses also exist. Even within a single tumor, identification of the genes involved

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in carcinogenesis is hampered by tumor heterogeneity and by the interaction of tumor cells with the underlying stroma.

Cancer research has traditionally focused on the roles of individual genes in carcinogenesis. However, the study of single genes has several limitations. The process of single-gene investigation can be biased as well as labor- and time-intensive. Advances in technology, however, now allow for the study of the entire exome or genome. The study of the cancer genome elucidates pathways and other complex interactions that may not be apparent at the level of single genes. Whole exome/genome approaches permit the unbiased assessment of which genes and pathways have been altered. All known human genes may now be evaluated in large numbers of tumors, resulting in a more comprehensive understanding of the complex changes that occur in the formation and progression of cancer ³.

In this review, we briefly describe the recent technological advances that have allowed for whole-exome/genome analysis of genetic and epigenetic alterations, as well as changes in gene expression profiles. We also describe some of the genes that have been implicated in HNSCC using these techniques. Finally, we explore implications for therapy as well as future directions for the field.

Genetic alterations

Genetic alterations in cancer may occur in the form of small intra-genic mutations, such as point mutations and insertions/deletions, or large alterations including genomic deletions, amplifications, and chromosomal rearrangements. Whole cancer exomes/genomes can be evaluated for genetic aberrations using either next-generation sequencing (NGS) or comparative genomic hybridization (CGH) technologies.

Next-generation sequencing

Next-generation sequencing technology allows for massively parallel sequencing, producing data with greater speed and at lower cost compared to more traditional methods. NGS instruments can process up to several million sequence reads in parallel, compared to the 96 reads produced by capillary-based instruments. In addition, template preparation, sequencing, and imaging steps for NGS platforms are highly automated and streamlined, requiring less time and additional equipment than high-throughput capillary-based sequencing systems ^{8,9}.

Next generation sequencing has several other advantages over capillary-based methods such as Sanger sequencing. Sanger sequencing has generally been limited to the analysis of either single genes or select “hot-spot” regions within the genome. In contrast, NGS allows the detection of base substitutions, deletions, insertions, copy number variations, and chromosomal translocations. NGS technologies have increased sequencing rates by several orders of magnitude and significantly reduced the cost per base, making it possible to sequence all known genes in multiple tumors of a given cancer type or in matched tumor and normal tissues ⁸⁻¹⁰.

Sequencing of either whole exomes or genomes can be performed using NGS platforms. Protein-coding regions constitute only about 1% of the human genome but are thought to account for 85% of mutations resulting in disease ¹¹. Since it targets that part of the genome enriched for causative genes, whole-exome sequencing is efficient, affordable, and allows many more samples to be sequenced. In addition, due to exome enrichment and higher base coverage, whole-exome platforms are currently more sensitive than whole-genome technologies for the detection of variants within coding regions ¹². However, whole-exome sequencing cannot identify variants in non-coding regions or genomic structural variations,

whereas whole-genome sequencing can identify both. Although greater genomic coverage may be useful, whole-genome sequencing generates vast amounts of data of yet unknown functional and clinical significance.

Recently, two studies were published in which whole-exome sequencing was performed in a total of 106 primary HNSCC tumors with matched normal DNA. Mutations were confirmed in several genes, including in *TP53*, *CDKN2A*, *FAT1*, *PTEN*, *HRAS*, *PIK3CA*, and *EGFR* that had been previously implicated in HNSCC. Both studies also identified mutations in *NOTCH1*, which had never previously been associated with HNSCC (Figure 1)^{7,13}.

We briefly review the most commonly mutated genes in HNSCC, as identified in these two studies (Table 1).

Tumor Suppressor Genes

TP53: The NGS studies confirmed the well-established role of *TP53*, a tumor suppressor gene on chromosome 17p12, in HNSCC. Mutated in approximately half of all HNSCC tumors^{7,13–15}, *TP53* is the most commonly mutated gene in HNSCC. Functional loss of p53 has been demonstrated in many human cancers and plays a critical role in malignant transformation^{16,17}. In fact, in the carcinogenesis of HNSCC, mutations in *TP53* occur early. *TP53* mutations are present in dysplastic premalignant lesions of the oral cavity, and the prevalence of these mutations increases with histopathologic progression of the tumor from dysplasia to invasive carcinoma^{18,19}.

Under normal circumstances, in response to DNA damage, p53 accumulates within the nucleus and causes cell cycle arrest via transcriptional induction of downstream effectors^{20,21}. If DNA repair is unsuccessful, p53 triggers apoptosis or senescence²¹. However, cells harboring mutations in *TP53* will not undergo cell cycle arrest, apoptosis, or senescence. The p53-deficient cells can replicate in the presence of damaged DNA and accumulate additional genetic mutations, leading to unchecked cell division and tumor formation and progression.

TP53 mutations in HNSCC have been associated with poor clinical outcomes, as well as poor response to treatment. A large prospective multicenter trial including 420 patients found that mutations disruptive to the DNA-binding domain of p53 decreased overall survival by 1.7 times compared to patients without disruptive mutations¹⁴. Poor tumor response to radiation or chemotherapy has also been associated with mutations in *TP53*. Alterations of p53 by mutation, deletion, or other mechanisms of inactivation have been found in 95% of HNSCC tumors refractory to radiation²². The risks of loco-regional recurrence and death after either primary or post-operative radiation therapy have both been found to be significantly greater for patients with mutations in *TP53*^{23–25}. *TP53* mutations have also been associated with poor response to cisplatin and fluorouracil²⁶. In a prospective study of 106 patients with HNSCC, *TP53* mutations were more frequent in patients who did not respond to cisplatin and fluorouracil than in patients who did respond. *TP53* mutation status was found to be an independent predictor of response to chemotherapy²⁷.

NOTCH1: *NOTCH1* is the second most commonly mutated gene in HNSCC, with a mutation rate of 14 to 15%^{7,13}. *NOTCH1* is important in regulating normal cell differentiation, lineage commitment, and embryonic development²⁸. It appears to function as a tumor suppressor gene in HNSCC based on the position and characteristics of the mutations and the inactivation of both alleles⁷. *NOTCH1* is thought to act as a tumor suppressor gene in several other human cancers, including cutaneous SCC²⁹, lung SCC³⁰,

and chronic myelomonocytic leukemia³¹, though it appears to function as an oncogene in other leukemias³².

The NOTCH1 protein is a transmembrane ligand receptor with intracellular and extracellular domains. Upon ligand binding, the NOTCH1 intracellular domain (NICD) is cleaved and translocates to the nucleus. In the nucleus, the NICD activates transcription by binding to CBF1 in the presence of co-activators from the Mastermind-like family (MAML). Downstream target genes of NOTCH1 signaling are crucial for cell differentiation and normal embryonic development²⁸.

Activating and loss-of-function mutations preferentially occur in different regions of the *NOTCH1* gene (Figure 2). Most *NOTCH1* mutations observed in HNSCC affect the epidermal growth factor (EGF)-like ligand-binding domain and are thought to lead to loss of function⁷. Inactivating mutations in these regions of the gene have also been reported in skin and lung SCC³⁰. In contrast, mutations of the intracellular PEST regulatory domain or the extracellular heterodimer domain are thought to result in constitutive activation of NOTCH1 signaling³³.

Mutations in the gene *FBXW7* have also been identified in 5% of HNSCC specimens⁷. *FBXW7* is a member of the F-box protein family and is a component of the ubiquitin ligase complex that can mediate NOTCH1 degradation. *FBXW7* mutations could therefore also affect the *NOTCH1* pathway, although *FBXW7* is also known to target other oncogenic pathways, such as cyclin E and c-myc³³.

CDKN2A: Alterations of cyclin-dependent kinase inhibitor 2A (*CDKN2A*/p16^{INK4A}), a tumor suppressor gene located on chromosome 9p21, have long been recognized in HNSCC^{34,35}. In the NGS studies, *CDKN2A* mutations were identified in 9 to 12% of all tumors^{7,13}. Gene copy number analyses also revealed frequent loss of heterozygosity and deletions of *CDKN2A*⁷. In addition to deletions and point mutations, *CDKN2A* is also inactivated by methylation of the 5' CpG region³⁶.

The protein product of *CDKN2A*, p16, plays a critical role in cell cycle regulation via its interaction with the retinoblastoma (Rb) tumor suppressor. The p16 protein inhibits cyclin-dependent kinases (CDK) 4 and 6, which are in turn necessary for the phosphorylation of Rb. Hypophosphorylated Rb induces G1 arrest of the cell cycle³⁷⁻³⁹.

Alterations of *CDKN2A*, though common events in early HNSCC development, alone are likely insufficient to drive tumorigenesis. This is supported by the fact that *CDKN2A* mutations have been reported in benign epithelial lesions with low potential for malignant transformation⁴⁰.

FAT1: *FAT1* is a tumor suppressor gene in the cadherin family of integral membrane proteins and is involved in cell adhesion, migration, and invasion⁴¹. It was found to have a 12% incidence of mutation¹³. In previous studies, homozygous deletions in *FAT1* were identified in the majority of oral SCCs⁴².

PTEN: *PTEN* is a negative regulator of PI3K, and its loss results in activation of the PI3K/Akt pathway, which is discussed below. Inactivating mutations were found in *PTEN* in 7% of tumors¹³, consistent with previous reports of mutation rates as high as 10%⁴³.

Oncogenes

HRAS: The true incidence of *Ras* mutations in HNSCC has been unclear. The reported frequency of mutations in the *Ras* family gene *HRAS* in HNSCC has varied from 0% in

Western populations to as high as 35% in Indian populations^{44–46}. These differences in mutation frequencies are thought to be related to the use of tobacco chewing and betel quid habits in Indian and other Asian countries, though it may also reflect underlying genetic variations between ethnic groups⁴⁷. Both of the NGS projects confirmed the presence of *HRAS* mutations in HNSCC, with a frequency of 4 to 5%^{7,13}.

Ras proteins are GTPases that function as signaling switches by alternating between the GTP-bound active state and the GDP-bound inactive state⁴⁸. Ras downstream effector pathways include Raf. Activated Raf phosphorylates MEK, which in turn activates ERK. The Raf/MEK/ERK pathway is involved in the regulation of cell proliferation, differentiation, and survival^{49,50}.

PIK3CA: Mutations were identified in the oncogene *PIK3CA* in 6 to 8% of tumors^{7,13}. Previous studies have found rates of *PIK3CA* mutation as high as 20%⁵¹. *PIK3CA*, which encodes the catalytic subunit p110alpha of the PI3K heterodimer, activates the AKT/mTOR pathway and promotes cell growth, cell survival, transformation, and drug resistance⁵².

EGFR: The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that belongs to the ErbB family of cell surface receptors and is involved in cellular proliferation, apoptosis, invasion, angiogenesis, and metastasis via the MAPK, AKT, ERK and JAK/STAT pathways^{53–55}. Focal amplification of 7p, which contains the *EGFR* gene, was found in approximately 10% of samples by copy number analysis⁷. Dysfunction of the EGFR pathway has been described in 80–90% of HNSCC, with over-expression of EGFR being the most common cause of dysregulation^{54,56,57}.

Comparative genomic hybridization

Variations in chromosomal structure, including inversions, deletions, translocations, or gains or losses of entire chromosomal segments, are common in the development and progression of HNSCC⁵⁸. Resultant DNA copy number alterations may change gene expression and function, resulting in tumor development.

The most commonly described chromosomal aberrations in HNSCC include: 4,7,59–62

- gains in 1q, 3q, 5p, 7p, 8q, 9q, 11q, 14q, and 18p
- losses in 3p, 4p, 4q, 5q, 8p, 9p, 10p, 11q, 13q, 17p, 18q, and 21q

Many of these genomic gains and losses were originally detected using loss of heterozygosity analysis or traditional cytogenetic techniques such as karyotype analysis. However, karyotype analysis is technically challenging to perform in solid tumors and, furthermore, does not allow detection of submicroscopic losses or rearrangements⁶³.

CGH permits the efficient analysis of the entire genome for DNA copy number variation—However, the minimum size of a detectable segment in standard CGH is 3 to 5 Mb⁶⁴, which limits detection of smaller alterations and makes identification of specific candidate genes difficult. More recently, microarray-based assays, or array CGH, have been developed to overcome the pitfalls of karyotype and standard CGH-based analyses. Array CGH, which utilizes bacterial artificial chromosome (BAC) arrays, allows for the high-throughput analysis of DNA copy number variations throughout the whole genome and allows high-resolution mapping of these changes directly onto genomic sequence^{2,59}. The resolution of array CGH platforms continues to improve to sub-megabase levels, so that variations ranging from gene-size aberrations to entire chromosomal arms may now be detected⁶⁵. Of note, whole-genome analysis of copy number variation can also be assessed using newer single nucleotide polymorphism (SNP) array platforms, which have the

additional advantage of being able to detect loss of heterozygosity ⁶⁶. However, to date, the majority of whole-genome studies of copy number variation in HNSCC have used BAC-based array CGH (Table 2).

Array CGH has been used in several studies to identify candidate pathways and genes in HNSCC tumorigenesis—Sparano et al. identified 22 amplified and 17 deleted genes in at least 25% of oral SCC ⁶⁷. Snijders et al. used array CGH to identify several common regions of chromosomal amplification containing genes involved in integrin signaling, adhesion, migration, and survival pathways in oral SCC. They also found dysregulation of the Hedgehog and Notch pathways ⁶⁸. In another study, array CGH was used to identify a novel gene possibly involved in oral SCC tumorigenesis. Two neighboring regions on chromosome 8q were found to be amplified, one of which harbored a novel putative oncogene, *LRP12*. Over-expression of *LRP12*, but not of two flanking genes, was then confirmed using RT-PCR ⁶⁹.

Array CGH has been used to attempt to delineate differences in the molecular and clinical characteristics of tumors—Smeets et al used array CGH to compare HPV-positive and HPV-negative tumors. The two subsets of tumors were found to harbor different genomic gains and losses, though some genetic changes were shared by both tumor types. This provides evidence of differences in the genetic alterations observed in HPV-positive compared to HPV-negative tumors ⁷⁰.

Associations between clinical outcomes and specific structural variations have also been identified. A study using standard CGH in HNSCC identified significant associations between gain of 3q25-q27 and loss of 22q with reduced disease-specific survival, as well as gain of 17q and 20q and deletion of 19p and 22q with reduced disease-free survival ⁷¹. A more recent study conducted array CGH in advanced stage oral SCC and found that gain of region 11q22.1-q22.2 and losses of 17p13.3 and 11q23-q25 were significantly associated with earlier loco-regional recurrence and shorter overall survival ⁷². Uchida et al. found that loss of 3p26.3 was significantly associated with poorer disease-specific survival ⁷³. Another study demonstrated an association between amplification at two distinct regions of 11q13 and metastases to the cervical lymph nodes ⁷⁴.

CGH has also been used to predict differences in response to treatment, though such studies have not yet been performed using array-based approaches. Akervall et al demonstrated that cisplatin-resistant cell lines had higher rates of chromosomal gains and losses compared to cisplatin-sensitive cell lines ⁷⁵. Another study used standard CGH to compare chemoradiotherapy-resistant primary HNSCC tumors to sensitive tumors. Both groups had similar total numbers of genetic changes, but high-level DNA amplifications were more frequent in the resistant tumors. Several specific chromosomal gains and losses were significantly associated with resistance to treatment. Such genomic profiles may be useful as predictors of resistance to chemoradiotherapy ⁷⁶.

Epigenetic alterations

Epigenetic regulation of gene expression occurs through several mechanisms, including DNA methylation, histone modification, and RNA interference by microRNA and small interfering RNA. Promoter methylation in particular is thought to play a role in the carcinogenesis of many human cancers ⁷⁷. Regions rich in cytosine-guanine dinucleotides, known as CpG islands, exist throughout the genome, typically within or upstream of promoter regions. CpG islands are found in up to half of all human genes ⁷⁸. Methylation occurs at the 5' carbon of the cytosine ring, which results in the recruitment of methyl-CpG binding domain proteins and histone deacetylases that inhibit binding of RNA polymerase.

Gene expression is thereby silenced, even if the DNA coding sequence remains unchanged ⁷⁹.

Epigenetic regulation of several genes, such as *CDKN2A*, *CDH1*, *MGMT*, and *DAPK1*, has been demonstrated in HNSCC ^{78,80}. However, most studies to date in HNSCC have analyzed the methylation status of individual genes previously implicated in carcinogenesis. Several techniques have been used to evaluate genome-wide methylation, including restriction landmark genomic scanning (RLGS), methylation-specific restriction enzyme (MSRE) analysis, and arbitrarily-primed PCR ^{59,78}.

The first genome-wide analysis of promoter methylation in HNSCC utilized RLGS, which uses gel electrophoresis to determine methylation status (Table 3). This study analyzed 1,300 CpG islands in 13 matched primary and metastatic HNSCC tumors. Global hypermethylation was found in the metastatic tumors compared to primary tumors ⁸¹. The first study utilizing array-based technology was performed by Adrien et al MSRE analysis was used to assess more than 12,000 CpG sites in 37 HNSCC tumors. Unsupervised hierarchical clustering yielded three distinct DNA methylation profiles among the tumors, although no correlations between methylation profiles and tumor or patient characteristics were identified ⁸².

Several methylation microarray platforms are now commercially available. Such technologies permit the rapid assessment of global methylation, as well as the identification of specific epigenetically regulated genes. One study identified a candidate gene family that may be involved in carcinogenesis by querying over 27,000 CpG sites in 24 oropharyngeal tumors and matched normal tissues. They identified 958 loci that were differentially methylated between tumors and normal tissues. A number of these loci were located on chromosome 19 and were associated with Kruppel-type zinc finger protein genes. Further analysis of these genes with quantitative RT-PCR confirmed decreased gene expression, although the clinical relevance of these changes in gene expression was not assessed ⁸³.

Methylation arrays have also been used to generate methylation profiles that may be used as biomarkers for disease or prognosis. One study identified a methylation profile of six CpG loci that differentiated patients with HNSCC from those without cancer ⁸⁴. Poage et al. found that promoter hypermethylation at two specific CpG sites, associated with the genes *TAP1* and *ALDH3A1*, was significantly correlated with decreased overall survival. This study not only identified two genes for possible therapeutic intervention but also delineated a methylation profile that may serve as a biomarker of more aggressive disease ⁸⁵. Therefore, methylation profiles using DNA obtained from minimally invasive methods may eventually be used in the clinical setting for diagnostic and screening purposes.

Altered gene expression profiles

Though sometimes used for gene discovery, gene expression arrays are most commonly used to establish expression profiles to serve as biomarkers that may distinguish tumors from normal or pre-malignant samples, identify subgroups of tumors, or predict clinical behavior ^{3,4,59}.

Several studies have identified gene expression profiles that differ between normal mucosa, premalignant lesions, and invasive carcinoma (Table 4). Data demonstrate that premalignant and malignant lesions cluster together, apart from normal mucosa, supporting the notion that altered patterns of gene expression most often occur before the development of malignancy ^{86,87}. Multiple groups have established specific gene panels that may be used to distinguish normal tissues from malignant lesions. For example, one study established a signature of 25 genes that can differentiate between oral SCC tumors and normal specimens.

This signature achieved 96% predictive accuracy on a cross-validation study and an average of 87% accuracy on three independent validation sets ⁸⁸. Other studies in oral SCC have defined gene sets to distinguish oral leukoplakia from invasive carcinoma. Kondoh et al. identified 11 genes that distinguished oral SCC from leukoplakia with greater than 97% accuracy ⁸⁹. Another group identified a panel of 9 genes that differentiated normal mucosa, oral leukoplakia, and invasive carcinoma ⁶². The results of each of these studies differ, likely due to differences in patient selection, tumor heterogeneity, and the use of different microarray platforms. However, their findings demonstrate the possibility that expression profiles may eventually be used as biomarkers in the clinical setting, to assist in diagnosis or disease monitoring.

Data from expression arrays have also been used to classify tumors based on their molecular characteristics. For example, Chung et al. identified four subtypes of HNSCC, each with a distinct gene expression pattern and different recurrence-free survival rates ⁶. Several groups have also identified differences in gene expression between HPV-positive and HPV-negative tumors. One study found differential expression of 347 genes in HPV-positive compared to HPV-negative tumors. Of note, some of the differentially expressed genes, including *CCND1* and *TYMS*, play roles in sensitivity to cisplatin and fluorouracil, while another gene, *RBBP4*, plays a role in sensitivity to radiation ⁹⁰. The results of these studies may therefore elucidate some of the molecular mechanisms of HPV-related tumorigenesis and response to therapy and may help guide therapeutic decision-making.

Several studies have identified specific gene expression profiles that may predict disease progression and clinical outcomes. Two studies analyzed gene expression profiles of primary tumors that had or had not metastasized to the cervical lymph nodes. The first study identified a panel of 8 genes with predictive accuracy of 92.3% ⁹¹, and the second identified a panel of 102 genes with 86% accuracy ⁹². Another study looked specifically at primary oral tongue SCC tumors in patients with cervical lymph node metastases, extra-capsular spread, or both. They identified distinct gene panels of three genes each that could predict either nodal metastasis or extra-capsular spread with 100% sensitivity and specificity by analysis of the primary tumor ⁹³.

Attempts have also been made to define expression profiles that can predict response to chemotherapy or radiation. A small prospective study of 14 patients with HNSCC found differential expression in 142 genes between tumors that were responsive or resistant to radiation therapy with or without chemotherapy. The gene panel showed greater than 93% accuracy for prediction of treatment response ⁹⁴.

Implications for Therapy

The biological complexity of HNSCC has become even more apparent with advances in our understanding of cancer genomics. The disease represents a heterogeneous collection of tumors in which multiple genes and pathways are altered. As evident in this review, aberrations may occur via a combination of genetic and epigenetic mechanisms affecting any number of genes and pathways. More sophisticated analyses of the pathways implicated in HNSCC tumorigenesis are therefore critical in the development of new targeted therapies and individualized medicine.

Knowledge gleaned from recent genomic studies offers new promise for the treatment of HNSCC. In particular, several therapies are currently being evaluated that target some of the genes found to be mutated by whole-exome sequencing ^{7,13}.

Gene therapies aimed at restoring wild-type *TP53* have shown promise in the treatment of patients with HNSCC. Wild-type *TP53* is incorporated into adenoviral vectors and injected

into the tumor, resulting in expression of normal p53 protein within tumors^{95,96}. ONYX-015, another adenoviral-based therapy that has oncolytic effects specifically in cells lacking functional p53, has recently been approved for use in China⁹⁶.

Targeting *NOTCH1* for therapeutic intervention is complicated by the fact that the gene may have either tumor suppressor or oncogenic functions depending on the cellular context. Several gamma-secretase inhibitors (GSI) block proteolytic activation of NOTCH1^{32,97}. GSIs are currently being evaluated in human trials for treatment of T-cell acute lymphoblastic leukemia and breast cancers^{98,99}. However, successful inhibition of *NOTCH1* oncogenic activity at one site may result in loss of its tumor suppressor function at another site. A phase III trial of GSIs for the treatment of Alzheimer's disease was recently halted due to an elevated incidence of skin cancer in treated patients, possibly through adverse effects on squamous epithelial differentiation¹⁰⁰. Further investigation into the functions of *NOTCH1* in different cell types is therefore necessary for the development of new targeted treatments.

Several therapies targeted against *HRAS* have been studied.

Farnesyltransferase inhibitors (FTI) prevent localization of Ras proteins to the cell membrane, thereby preventing signal transduction. FTIs have shown some antitumor effect in early clinical trials in several cancers, including multiple myeloma, lung cancer, and some leukemias¹⁰¹. In addition, the use of antisense oligonucleotides directed against *HRAS* mRNA has been studied in solid tumors^{102,103}. However, a phase II clinical trial in pancreatic adenocarcinoma showed no additional benefit of the antisense compound beyond standard therapy¹⁰⁴. Overall, strategies to inhibit Ras signaling have shown limited efficacy in clinical trials, possibly due to secondary alterations in upstream and downstream Ras pathway effectors¹⁰⁵.

Therapies directed against *PI3KCA* and its downstream effectors have also been investigated. The addition of PI3K pathway inhibitors to standard therapy is being studied as an approach to overcoming resistance to conventional chemoradiotherapy. The PI3K inhibitor PX-866 is now in phase I and II trials in individual combinations with docetaxel or cetuximab for the treatment of HNSCC^{106,107}. PI3K has many downstream effectors, including Akt and mTOR, which may be targeted. The mTOR inhibitor rapamycin, an FDA-approved immunosuppressant, is in phase I trials for treatment-naïve advanced HNSCC patients¹⁰⁸, while an Akt inhibitor, MK2206, is currently in phase II trials for treatment of recurrent and metastatic HNSCC¹⁰⁹.

Lastly, therapeutic targeting of *CDKN2A* is limited by the challenge of restoring tumor suppressor activity. Therefore, strategies are instead aimed at inhibiting downstream targets that have been rendered overactive, such as CDKs. Results of phase II and III clinical trials of several first-generation CDK inhibitors have been disappointing, but several second-generation CDK inhibitors, which are thought to be more potent, are in advanced preclinical or clinical trials¹¹⁰.

We have reviewed a number of genomic signatures, at the levels of DNA sequence, methylation, and gene expression, which may serve as potential biomarkers in HNSCC. Specific tumor profiles may be used in the clinical setting for confirming diagnoses, predicting prognosis, tracking recurrence, or monitoring therapeutic response. Detection of these biomarkers in readily available sources like saliva or peripheral blood may be of particular value for early detection or disease surveillance.

Future Goals for Genomics studies

The advent of genomic technologies discussed in this review has resulted in the rapid generation of knowledge in our understanding of HNSCC. Genome-wide studies have identified aberrant genes and pathways that may play an active role in tumorigenesis, as well as potential biomarkers that may serve as indicators of disease state. However, these studies have thus far been largely descriptive, and much remains unknown about the specific molecular mechanisms underlying tumor formation and progression. Furthermore, due to discrepancies between studies in tumor characteristics and treatments, it is difficult to draw consistent, wide-ranging conclusions. The quality of available clinical data is also widely variable. More standardized, prospective collection of specimens and of clinical data is necessary in order to identify associations between specific genetic alterations and prognosis or other clinical outcomes.

Other genomic tools include next-generation sequencing of the whole genome and RNA (RNA-seq). As noted above, NGS detects base substitutions, insertion/deletions, and chromosomal inversions or translocations. NGS can therefore be used to identify chromosomal variations with very high resolution and is a useful adjunct to conventional or array CGH. RNA-seq will permit direct analysis of the complete transcriptome and will greatly increase the resolution of gene expression data beyond the limits of available expression microarrays. Data obtained from RNA-seq can also be integrated with data from other platforms to give insight into gene expression in the context of known mutations, DNA copy number alterations, or other genomic aberrations. RNA-seq can also be used to detect alternative splicing isoforms and fusion transcripts ⁹.

Genomic tools offer insight into the many genetic and epigenetic changes occurring in a single tumor. Emphasis will increasingly be placed on integrated pathway analysis, resulting in a better understanding of the complex interactions that occur within tumor cells. In addition, the integration of multiple platforms, such as gene expression profiling, methylation arrays or array CGH experiments, will allow us to answer increasingly sophisticated questions regarding the molecular underpinnings of HNSCC.

Ultimately, the goals of these investigations are to provide new tools to be used for diagnosis or disease monitoring and to uncover new targets for therapeutic intervention. Further research may help to ultimately realize the possibilities of targeted therapies and personalized medicine.

Summary

The advent of genomic technologies has greatly advanced our knowledge of the molecular changes underlying HNSCC. Technologies including next-generation sequencing and array-based platforms have provided us with new insight into the genes and pathways that may be altered in this disease. These innovations offer new targets for therapeutic intervention and new options for diagnosis and surveillance of the disease. However, as underscored by the findings outlined in this review, HNSCC is a complex and heterogeneous disease that we are only beginning to understand. Genomic approaches will hopefully continue to support the development of diagnostic or prognostic indicators and targeted therapies for our patients.

Abbreviations

BAC	bacterial artificial chromosome
CDK	cyclin-dependent kinases

CGH	comparative genomic hybridization
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FTI	Farnesyltransferase inhibitors
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
MAML	Mastermind-like family
MSRE	methylation-specific restriction enzyme
NGS	next-generation sequencing
NICD	NOTCH1 intracellular domain
Rb	retinoblastoma
RLGS	restriction landmark genomic scanning
SNP	single nucleotide polymorphism

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Key Points

- The study of HNSCC tumor development and progression is complicated by the biological complexity and heterogeneity of the disease.
- Recent technological advances now permit the study of the entire cancer genome, which can elucidate complex pathway interactions that are not apparent at the level of single genes.
- Next-generation sequencing technology allows for detection of base substitutions, deletions, insertions, copy number variations, and chromosomal translocations of entire exomes or genomes.
- Two recent whole-exome sequencing studies reported frequent mutations in *TP53*, *NOTCH1*, *CDKN2A*, *PIK3CA*, and *HRAS* in HNSCC tumors.
- Standard or array-based comparative genomic hybridization can detect variations in chromosomal structure with greater resolution than traditional cytogenetic techniques.
- Methylation and gene expression arrays can be used for gene discovery or for identification of tumor-specific profiles that may serve as biomarkers with diagnostic or prognostic value.

Figure 1.

Frequencies of genetic alterations identified by whole-exome sequencing and copy number analysis studies (7, 13). CNV: Copy number variation.

Figure 2.

Schematic depiction of mutations in *NOTCH1* noted in Agrawal et al. (7). (A) Previously observed *NOTCH1* mutations in hematopoietic malignancies. EGF, epidermal growth factor; LNR, Lin12-Notch repeats; TMD, transmembrane domain; RAM, recombination signal-binding protein 1 for J-k (RBPjk) association module; NLS, nuclear localization signal; PEST, proline, glutamic acid, serine/threonine-rich motifs. Red bars represent previously described mutation hotspots (amino acids 1575 to 1630 and 2250 to 2550). (B) Previously observed *NOTCH1* mutations in solid tumors. Colored arrow (missense mutation) and “X” (truncating mutation) depict mutations found in different tumor types: pink, breast cancer; black, glioma; blue, lung cancer; green, pancreatic adenocarcinoma; red, esophageal squamous cell carcinoma; purple, tongue squamous cell carcinoma. (C) Mutations in *NOTCH1* in HNSCC observed in this study. Black arrow indicates missense mutation and red “X” indicates truncating mutation. This figure has previously been published in Agrawal et al. (7).

Table 1

Frequently altered genes in HNSCC

Gene Symbol	Gene Name	Chromosomal Location	Gene Function	Mutation Rate	Copy Number Variation	References
Tumor Suppressor Genes						
<i>TP53</i>	Tumor protein p53	17p13.1	Tumor suppressor. Assists in cell cycle arrest, DNA damage repair, apoptosis, and senescence.	40–62%	N/A	7, 13–27
<i>NOTCH1</i>	Notch1	9p34.3	Tumor suppressor or oncogene (tissue-dependent). Important in regulation of cell differentiation, lineage commitment, and embryonic development.	14–15%	N/A	7, 13, 28–33
<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A	9p21.3	Tumor suppressor. Important in cell cycle regulation.	9–12%	29%	7, 13, 34–40
<i>FAT1</i>	FAT tumor suppressor homolog 1 (Drosophila)	4q35.2	Tumor suppressor. Member of cadherin family. Involved in cell adhesion, migration, and invasion.	12–80%	N/A	13, 41, 42
<i>PTEN</i>	Phosphatase and tensin homologue	10q23.3	Tumor suppressor. Negative regulator of PI3K.	7–10%	N/A	13, 43
<i>FBXW7</i>	F-box and WD repeat domain-containing protein 7	4q31.3	Tumor suppressor. Member of the F-box protein family and component of the ubiquitin ligase complex that can mediate NOTCH1, cyclin E and c-myc degradation.	5%	N/A	7, 33
Oncogenes						
<i>HRAS</i>	Harvey rat sarcoma viral oncogene homolog	11p15.5	Oncogene. GTPase important in promoting cell proliferation, differentiation, and survival through downstream effector pathways.	4–35%	N/A	7, 13, 44–50
<i>PIK3CA</i>	Phosphoinositide-3-kinase catalytic alpha polypeptide	3q26.32	Oncogene. Catalytic subunit of PI3K. Target of Ras activation. Promotes cell growth, survival, and cytoskeleton reorganization.	6–8%	10%	7, 13, 51, 52
<i>EGFR</i>	Epidermal growth factor receptor	7p12	Oncogene. Receptor tyrosine kinase in the ErbB family. Involved in cell proliferation, apoptosis, invasion, angiogenesis, and metastasis.	N/A	10%	7, 53–57

Abbreviations: N/A: Not applicable

Table 2
Selected studies using standard or array comparative genomic hybridization in HNSCC

Author, year	Methodology	Cohort	Findings	Reference
Snijders et al., 2005	In-house HumArray2.0 (UCSF)	89 oral SCC	Identified 9 recurrent amplified regions <3 Mb, which contained genes involved in integrin signaling, adhesion, migration, survival, Hedgehog, and Notch pathways, which were amplified and overexpressed.	68
Garnis et al., 2004	In-house BAC array	22 oral SCC	Identified 5.3 Mb region of common amplification at 8q22 containing 16 known genes. Gene expression analysis revealed overexpression of LRP12, a novel putative oncogene.	69
Smeets et al., 2006	In-house BAC array	12 oral SCC, 12 oropharynx SCC	Compared HPV-positive and HPV-negative tumors. Four regions of gains or losses were unique to HPV-negative tumors. Seven regions of gains or losses were altered at high frequency in both tumor subsets.	70
Ashman et al., 2003	Standard CGH	10 oral or oropharynx SCC, 35 other HNSCC	Gain of 3q25-q27 and loss of 22q were associated with reduced disease-specific survival. Gains of 17q and 20q, loss of 19p and 22q, and amplification of 11q13 were associated with reduced disease-free survival.	71
Ambatipudi et al., 2011	Human Genome CGH Microarray 105K (Agilent)	60 oral SCC	Gain of 11q22.1-q22.2 and losses of 17p13.3 and 11q23-q25 were associated with earlier loco-regional recurrence and shorter overall survival.	72
Uchida et al., 2011	MacArray Karyo 4K (Macrogen)	50 oral SCC	Loss of a 0.2 Mb region at 3p26.3 was associated with reduced disease-specific survival.	73
Sugahara et al., 2011	Human Genome CGH Microarray 44K (Agilent)	54 oral SCC (22 with cervical LN metastasis, 32 without cervical LN metastasis)	Compared tumors that had or had not metastasized to the cervical lymph nodes. Two distinct regions of amplification at 11q13 were associated with cervical metastasis.	74
van den Broek et al., 2007	Standard CGH	34 oral or oropharynx SCC, 6 other HNSCC	Compared chemoradiotherapy-sensitive and resistant tumors. Gains of 3q11-q13, 3q21-q26.1, and 6q22-q27 and losses of 3p11-pter and 4p11-pter were associated with chemoradiotherapy resistance.	76

Abbreviations: BAC: bacterial artificial chromosome; CGH: comparative genomic hybridization; Mb: megabase; SCC: squamous cell carcinoma; HNSCC: head and neck squamous cell carcinoma; HPV: human papilloma virus; LN: lymph node

Table 3

Selected studies of genome-wide methylation status in HNSCC

Author, year	Methodology	Numbers of CpG sites, genes analyzed	Cohort	Findings	Reference
Smiraglia et al., 2003	RLGS	1,293, N/A	13 matched primary tumor and cervical LN metastasis (12 oral or oropharynx SCC, 1 other HNSCC)	Found global hypermethylation in metastatic LNs compared to primary tumors. However, different loci were methylated in LNs compared to primary tumors.	81
Adrien et al., 2006	MRSE analysis	12,288, N/A	6 oral SCC, 17 oropharynx SCC, 13 other HNSCC	Identified 3 distinct methylation profiles. No associations found between methylation profile and tumor or patient characteristics.	82
Lleras et al., 2011	Infinium HumanMethylation27 BeadChip (Illumina)	27,578, 14,495	24 oropharynx SCC, 24 matched normal mucosa	Identified 958 loci that were differentially methylated between tumors and normal tissue. Many of these loci on chromosome 19 were associated with Kruppel-type zinc finger protein genes. Gene expression analysis verified decreased expression of these genes.	83
Langevin et al., 2012	Infinium HumanMethylation27 BeadChip (Illumina)	27,578, 14,495	39 oral SCC, 35 oropharynx SCC, 18 other HNSCC, 92 cancer-free controls	Evaluated DNA from peripheral blood. Identified a methylation profile of 6 CpG loci that differentiated between HNSCC patients and controls.	84
Poage et al., 2012	Infinium HumanMethylation27 BeadChip (Illumina)	27,578, 14,495	46 oral SCC, 15 oropharynx SCC, 15 other HNSCC	Hypermethylation at two specific CpG sites, associated with the genes <i>TAP1</i> and <i>ALDH3A1</i> , was associated with decreased overall survival.	85

Abbreviations: RLGS: restriction landmark genomic scanning; MSRE: methylation-specific restriction enzyme; N/A: not available; SCC: squamous cell carcinoma; HNSCC: head and neck squamous cell carcinoma; LN: lymph node

Table 4

Selected studies using gene expression arrays in HNSCC

Author, year	Array platform	Number of genes analyzed	Cohort	Findings	Reference
Ziober et al., 2006	Affymetrix U133A	14,500	13 oral SCC, 13 matched normal mucosa	Identified a panel of 25 genes that differentiated between oral SCC and controls with 96% predictive accuracy on cross-validation and an average of 87% accuracy on three independent validation sets.	88
Kondoh et al., 2007	IntelliGene HS Human Expression CHIP (TaKaRa)	16,600	5 oral SCC, 5 leukoplakia	Identified a panel of 11 genes that differentiated oral SCC from leukoplakia with >97% accuracy.	87
Liu et al., 2011	Oligo GEArray Human Cancer Microarray (SuperArray)	440	3 oral SCC, 3 leukoplakia, 3 normal mucosa	Identified a panel of 9 genes that differentiated between normal mucosa, oral leukoplakia, and invasive carcinoma.	62
Lohavanichbutr et al., 2009	GeneChip Human Genome U133 Plus 2.0 (Affymetrix)	39,000	88 oral SCC, 31 oropharynx SCC, 35 normal mucosa	Compared HPV-positive and HPV-negative tumors. Found differential expression of 347 genes, including <i>CCND1</i> , <i>TYMS</i> , and <i>RBBP4</i> , which play roles in sensitivity to chemotherapy and radiation.	90
Nguyen et al., 2007	GeneChip Human Genome U133 Plus 2.0 (Affymetrix)	39,000	30 oral SCC (13 with cervical LN metastasis, 17 without cervical LN metastasis)	Compared primary tumors that had or had not metastasized to the cervical LNs. Identified a panel of 8 genes that could predict LN metastasis with 92.3% accuracy.	91
Roepman et al., 2005	Human Array-Ready Oligo set (Qiagen)	21,329	82 oral or oropharynx SCC (45 with cervical LN metastasis, 37 without cervical LN metastasis)	Compared primary tumors that had or had not metastasized to the cervical LNs. Identified a panel of 102 genes that could predict LN metastasis with 86% accuracy.	92
Zhou et al., 2006	GeneChip Human Genome U133 Plus 2.0 (Affymetrix)	39,000	25 oral tongue SCC (11 with cervical LN metastasis, 14 without cervical LN metastasis)	Compared primary tumors that had or had not metastasized to the cervical LNs. Also compared tumors that did or did not have extra-capsular spread. Identified 2 panels of three genes each that could predict nodal metastasis or extra-capsular spread with 100% sensitivity and specificity.	93
Dumur et al., 2009	GeneChip Human Genome U133 Plus 2.0 (Affymetrix)	39,000	8 oral or oropharynx SCC, 6 other HNSCC	Compared tumors that were sensitive or resistant to radiation. Identified a panel of 142 genes that could predict treatment response with >93% accuracy.	94

Abbreviations: SCC: squamous cell carcinoma; HNSCC: head and neck squamous cell carcinoma; LN: lymph node