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The molecular pathogenesis of head and neck squamous cell carcinoma

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Rb/INK4/ARF, and Notch, in tumor initiation, progression, and maintenance. The increasingly refined knowledge of HNSCC genetics, combined with ever-more-sophisticated animal models and newer drug targeting strategies, should promote novel therapeutic approaches and improved disease outcomes.

Clinical and epidemiological features

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer worldwide, affecting 600,000 new patients each year. In the United States, 50,000 new cases are diagnosed, and nearly 10,000 deaths are attributable to this disease annually. Despite their common origin in the squamous mucosa of the upper aerodigestive tract, there are several sources for the heterogeneity of HNSCC that complicate their consideration as a single disease entity. Anatomically, the head and neck region consists of several distinct structures (e.g., lip, tongue, nasopharynx, oropharynx, larynx, hypopharynx) with distinct microscopic features and lymphatic and venous drainage. As a result, treatment approaches and outcomes vary significantly among the different subsites. In addition, etiologic risk factors vary among the different subsites, and HNSCC can occur in young patients without any known risk factors. Biologic heterogeneity independent of subsite has been suggested by molecular analyses that have uncovered distinct classes of HNSCC with unique mRNA expression profiles or patterns of DNA copy number alterations that correlate with clinical behavior (1–4).

Although the most important risk factors are tobacco use and alcohol consumption, infection with high-risk types of human papilloma viruses (HPVs) has also been recognized as an increasingly important risk factor, particularly for oropharyngeal squamous cell carcinoma (OPSCC). Importantly, survival for patients with HPV-positive OPSCC is significantly better than for HPV-negative cancers, irrespective of treatment modality (5–8). Possible explanations include both host-intrinsic factors (healthier patients with fewer comorbidities) and tumor-intrinsic factors (distinct genetic pathogenesis yielding enhanced treatment sensitivity). The relatively good prognosis of HPV-related cancers highlights an unfortunate paradox — despite improvements in outcome for patients with oropharynx cancers, little or no improvement has been observed for patients with HPV-negative tumors over the past 30 years (9). This fact is attributable at least in part to our limited understanding of molecular pathways that both promote HSNCC pathogenesis and can be targeted therapeutically.

Linking pathways to genetics

Recent advances in our understanding of the molecular pathogenesis of HNSCC were provided by whole-exome sequencing (i.e., sequencing exons of all known protein-coding genes) conducted on a total of approximately 100 HNSCC specimens independently by two groups (10, 11). While the two studies analyzed etiologically similar tumors with related sequencing platforms, there was a five-fold difference in the average number of mutations reported per tumor. This difference likely reflects distinct bioinformatic and validation approaches used in the studies, and therefore a subset of identified changes may represent “passenger” mutations (as a result of increased mutation rates in cancer cells, or even mutation “miscalls”) rather than true “driver” mutations with an etiologic role in HNSCC. Nevertheless, several key findings were shared by these studies. This work, together with a large body of previous genomic and functional analyses of HNSCC, highlights the relatively small number of oncogenes targeted by activating mutations and supports the fundamental roles of tumor suppressor pathways including p53, Rb/INK4/ARF, and Notch in disease pathogenesis. These and other bona fide HNSCC cancer genes play major roles in at least four key functional pathways: cellular proliferation, squamous epithelial differentiation, cell survival, and invasion/metastasis, with many of the genes impacting more than a single pathway (Figure 1). These pathways are critical to the pathogenesis of HNSCC and, not surprisingly, reflect normal developmental programs within the stratified squamous epithelium. Given the paucity of driver oncogenes in HNSCC, targeting these pathways therapeutically represents a substantial and critical challenge for improving outcomes of this disease.

Cellular proliferation and p53/Rb/CDKN2A/CCND1

Mutation of the *TP53* tumor suppressor gene is the most common and among the earliest identified genetic alterations in HNSCC, occurring in more than half of all cases (2). As in other human cancers, missense mutations primarily within the DNA binding domain account for 75% of all mutations in the *TP53* gene and confer both dominant negative and poorly understood gain-of-function properties (12–15). In many of the remaining HNSCC tumors in which p53 is wild-type, p53 function may be inactivated by other mechanisms.

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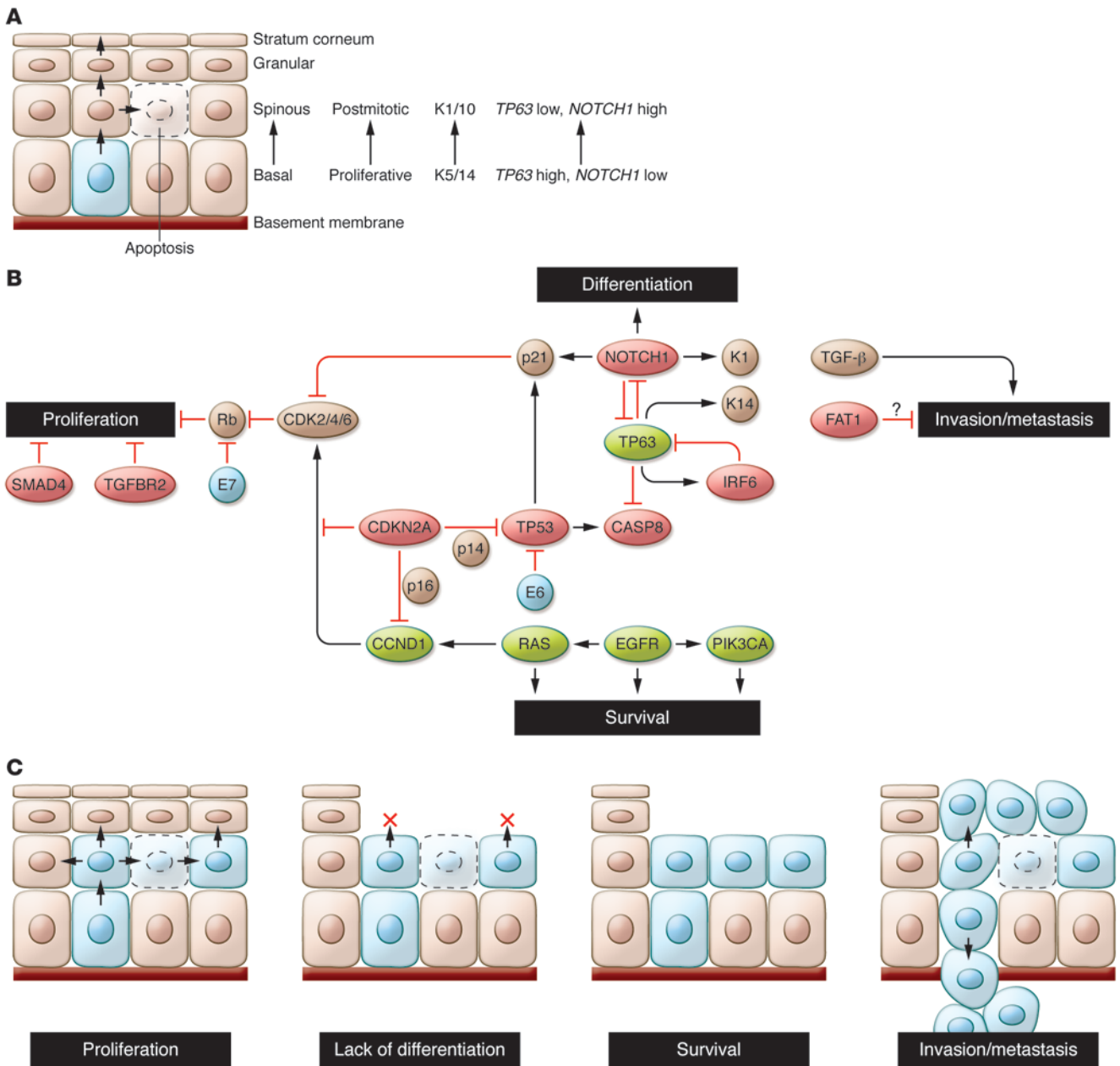


Figure 1

Hallmarks of head and neck squamous tumorigenesis. **(A)** The normal process of squamous morphogenesis in the adult mucosa is controlled in part by *TP63* and *NOTCH1*. The former is expressed in keratinocytes of the basal layer, where it maintains their proliferative potential and controls expression of basal markers (e.g., keratins 5/14 [K5/14]); expression of the latter results in terminal differentiation into cells of the spinous (K1/10) and granular layers. Rare stem cells in the basal layer (light blue) undergo terminal differentiation through asymmetric cell division. Abnormal proliferation is prevented primarily by differentiation-associated cell cycle exit and by apoptosis. **(B)** Pathways altered in HNSCC pathogenesis identified in whole-exome sequencing studies. Red: putative and established tumor suppressors; green: oncogenes; brown: other relevant genes/proteins; blue: viral proteins. Loss of *TP53* and *CDKN2A*, amplification of *CCND1*, and loss of *TGFB2/SMAD4* permit abnormal proliferation and decrease apoptosis. However, abnormal cell cycling may still be restrained by intact differentiation and apoptotic programs. Loss of *NOTCH1* and/or abnormal expression of *TP63*, together with alterations in “survival” genes (e.g., *CASP8*, *PIK3CA*, *EGFR*), may remove additional barriers to tumor cell proliferation and survival. Loss of cell adhesion genes (e.g., *FAT1*) could permit release of cells from the mucosal lining, while invasion through the basement membrane is promoted by *TGFB1* (and *SMAD3*). **(C)** Schematic of HNSCC hallmarks. The precise order of acquisition of distinct alterations is not clear. In addition, several genes (e.g., *TP53*, *TP63*, *NOTCH1*) may contribute to more than one hallmark.



These include expression of the HPV E6 protein (which binds p53 and targets it for proteasomal degradation), overexpression/amplification of *MDM2* (which also mediates p53 proteasomal degradation), and deletion of *CDKN2A*, which may eliminate p14/ARF, a negative regulator of *MDM2* (16–19). Overall, the data suggest that the p53 pathway is downregulated in at least 80% of HNSCCs (2).

The finding that *TP53* is mutated in both leukoplakia (a histologically recognizable precursor lesion) and benign-appearing mucosa has led to a “patch-field” progression model of HNSCC development, in which the index squamous carcinoma (as well as subsequent tumors) develops from a field of genetically abnormal mucosa, itself the result of expansion of a clonal patch arising from a putative stem cell containing a mutated *TP53* gene (20). Interestingly, in some cases the *TP53* mutations found in the tumor and adjacent mucosa are different, implying a distinct clonal origin for multiple patches and suggesting that metachronous tumors from the same patient (e.g., primary versus locally recurrent) could in fact develop from unique clones through independent acquisition of additional alterations (21). In addition to tumor initiation, *TP53* inactivation also contributes to the clinical behavior of tumors, at least in part independent of an influence on the response to genotoxic therapy. Thus, truncating and function-disrupting mutations of *TP53* are significantly associated with decreased survival — after primary surgery with or without postoperative radiotherapy — compared with either non-disruptive mutations or no mutation at all (22, 23).

The essential role of the retinoblastoma (Rb) pathway is evidenced by the finding of inactivation of *CDKN2A*, encoding the cell cycle regulators p16/INK4A and p14/Arf/INK4B, in HNSCC. *CDKN2A* mutations were found in approximately 7% of tumors by exome sequencing, with copy number losses in another 20%–30% (10, 11). It has been previously shown that *CDKN2A* inactivation by mutation is significantly more rare than deletion or epigenetic inactivation, which together account for inactivation of the gene in up to 75% of HNSCCs (24–26). Although p16/INK4A loss (whether genetic or functional) has been repeatedly demonstrated to correlate with indicators of worse prognosis, data on p14/Arf/INK4B loss (e.g., by methylation, when the genomic locus itself is not deleted) is conflicting, with one study suggesting worsened prognosis, while two others suggested improved prognosis, perhaps a result of increased radiation sensitivity (27–29). In the case of HPV+ HNSCC, inactivation of the Rb pathway is achieved through expression of the HPV E7 protein, which binds RB1 and abrogates the requirement for p16/INK4A silencing. As a result, assaying p16 protein expression in tumor cells by immunohistochemistry (IHC) is of clinical value in determining HPV+ status (30).

Amplification of a discrete, approximately 5-Mb region of chromosome 11q13 containing the *CCND1* gene (encoding cyclin D1) occurs in approximately one-third of HNSCCs, and perhaps even more frequently in HPV-negative tumors (4, 31). Furthermore, overexpression of cyclin D1 has been observed in up to 80% of HNSCCs (2). This high frequency is remarkable given that *CDKN2A* loss or *CCND1* amplification would seem to be redundant mechanisms to promote cell cycle progression through activation of G₁ phase cyclin-dependent kinases (CDKs) 4 and 6. Nevertheless, these two genetic events are not mutually exclusive in HNSCC, potentially reflecting either qualitatively or quantitatively different effects. For example, cyclin D1 may indirectly stimulate CDK2 activity by sequestering the CDK2 inhibitors p21 and p27, and alternatively, cyclin D1 may function as a cofactor independent of its role in cell

cycle regulation, through binding to transcription factors (e.g., PPAR γ) or DNA repair proteins (e.g., BRCA2, Rad51) (refs. 32, 33; reviewed in ref. 34). In keeping with their distinct contributions, loss of p16 expression and overexpression of cyclin D1 are independent predictors of death from tongue cancer, and the loss of p16 together with overexpression of cyclin D1 confers significantly worse 5 year survival than either condition observation alone (35).

Terminal differentiation and the Notch/p63 axis

Perhaps the most novel finding to emerge from the whole-exome sequencing studies of HNSCC is the discovery of mutations within the *NOTCH1* gene in 12%–15% of cases, and within additional *NOTCH* family members in 3%–5% (10, 11). Although Notch signaling had previously been implicated as pro-tumorigenic — by virtue of activating mutations and translocations observed in the genes for Notch receptors or their regulators in T cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, and diffuse large B-cell lymphoma (36–40) — several of the *NOTCH* family mutations in HNSCC (and in chronic myelomonocytic leukemia, a rare myeloproliferative disease) encode inactivating mutations, suggesting a tumor suppressor function (41). The physiologic relevance of these findings is supported by animal models in which *NOTCH* activation in hematopoietic cells leads to T cell leukemias and inactivation in squamous epidermis promotes skin tumorigenesis (refs. 42–44; reviewed in ref. 45).

Notch signaling has been linked to multiple biological functions, including regulation of self-renewal capacity, cell cycle exit (in part through upregulation of p21/CDKN1A expression), and cell survival (46–48). In the stratified epithelium, Notch has a central role in promoting terminal differentiation (48, 49), which is mediated through both direct effects (e.g., on activation of suprabasal keratins) and indirect effects on the Wnt, hedgehog, and interferon response pathways (43, 47, 50, 51). Additionally, Notch activity has been linked to suppression of HPV E6 and E7 protein expression, potentially providing additional selective pressure for loss of Notch in HPV+ HNSCC (52, 53).

Further supporting a role for Notch in squamous epithelial differentiation is its control by the p53-related transcription factor p63, a master regulator of proliferative potential, lineage specification, and differentiation in stratified epithelia. Constitutive knockout of *Tp63* in mouse models results in complete failure of normal epidermal development (54, 55). In mature epithelium, expression of p63 is highest in basal epithelial cells, where it functions as an inhibitor of NOTCH1 expression, and becomes downregulated during terminal differentiation coincident with NOTCH1 upregulation (Figure 1A and ref. 56). Reactivation of p63 expression is observed in the suprabasal layers of dysplastic mucosa, and overexpression and/or genomic amplification of the *TP63* locus is observed in the majority of invasive HNSCCs (57, 58). *TP63* gives rise to two major isoform classes, TAp63 and Δ Np63, which differ in the presence and absence, respectively, of an N-terminal transcriptional transactivation domain. Although tumor incidence data from *Tp63*-heterozygous mice are conflicting, Δ Np63 isoforms, which are selectively overexpressed in HNSCC, are likely oncogenic (59, 60). Importantly, *TP63* was found to be mutated or amplified in 8% of samples in one of the sequencing studies (11). Consistent with a contribution of Δ Np63 in these tumors, two of the mutations uncovered are predicted to alter the function of TAp63 (including a nonsense mutation) but not Δ Np63. In addition to its contribution through Notch suppression, Δ Np63 has been demonstrated to control other key



tumor-relevant pathways, including cell survival (in part through suppression of the proapoptotic p53-related protein p73), senescence suppression (through suppression of p16/INK4A expression), and growth factor signaling (through induction of EGFR) (61–64).

Cell survival through EGFR/Ras/PIK3CA/PTEN/CASP8

The PI3K signaling pathway is commonly activated in HNSCC, as evidenced by recurrent alterations of two central regulators: *PTEN*, encoding a negative regulator, and *PIK3CA*, encoding a positive regulator of this pathway. *PTEN* is subject to frequent loss of heterozygosity in a variety of cancers, including up to 40% of HNSCCs, although biallelic inactivation occurs less frequently (65–68). Loss of just a single *PTEN* allele in the remaining samples may contribute to tumorigenesis, however, since recent data suggesting a gene dosage effect for *PTEN* (69). Activating mutations in two “hot spot” regions of the *PIK3CA* gene occur in 6%–11% of HNSCCs, with a potential enrichment in tumors originating from the pharynx (70, 71). The latter finding is particularly noteworthy given the increased incidence of *PIK3CA* mutations in HPV-related versus non-HPV-related tumors observed in both exome sequencing studies. This observation suggests that *PIK3CA* mutations may cooperate with HPV E6 and E7 proteins in the development of invasive OPSCC, as has been suggested for cervical carcinomas (72). The prominent role of the PI3K pathway in HNSCC has potentially important clinical implications, given that numerous targeted inhibitors of this pathway are currently being evaluated in clinical trials (reviewed in ref. 73).

Activating missense mutations causing single amino acid substitutions in one of three positions (codon 12, codon 13, and codon 61) in the *HRAS* gene were uncovered in 3%–5% of samples in both whole-exome sequencing studies. While it is currently unknown whether *HRAS*-dependent signals function in collaboration with or independently of PI3K activation in HNSCC, several findings underscore the importance of this particular *RAS* family member to the pathogenesis of the disease. These include the more frequent occurrence of *HRAS* than *KRAS* mutations in HNSCC, particularly in relationship to tobacco history, whereas the reverse is true for several other malignancies (74, 75); the presence of *HRAS* mutations in HPV-driven tumors, suggesting potential cooperativity in tumor promotion (76); and the more frequent association of *HRAS* versus *KRAS* mutation in squamous cell carcinomas arising in the setting of tobacco exposure in humans and chemical carcinogen exposure in mice (77). Although Ras proteins themselves have proven difficult to target directly, therapeutic strategies that target downstream effectors of Ras proteins or the synthetic lethal dependencies that result from their mutational activation have already been successful in preclinical models (78–80).

Upstream signaling to both Ras and PI3K pathways may occur through activation or overexpression of receptor tyrosine kinases (RTKs) including EGFR. Although it is often considered to be among the most important therapeutic targets in HNSCC (81), our understanding of the role of EGFR is evolving with the appreciation that *EGFR* activating mutations are rare in HNSCC and that the reported frequency of *EGFR* gene amplification in HNSCC varies widely, in part due to varying definitions of the degree and size of copy number gain that constitute amplification (82, 83). Furthermore, although copy number gain of *EGFR* has been suggested to correlate with poor prognosis in HNSCC (83, 84), in general gain of *EGFR* has not been clearly demonstrated to predict improved outcomes following EGFR-directed therapy (85,

86). Similarly, therapeutic agents that inhibit EGFR, including the small molecule inhibitors gefitinib and erlotinib and the therapeutic antibody cetuximab, have modest activity in HNSCC, with little or no correlation with EGFR status (87–91).

Two other genetic abnormalities affecting RTK signaling have received less attention but have potential near-term clinical impact. Expression of EGFRvIII, a variant EGFR protein that results from the in-frame genomic deletion of exons 2–7 and is present in gliomas and lung squamous cancers, was recently reported in 42% of HNSCCs (92). Importantly, an antibody thought to be specific for EGFRvIII (e.g., rather than full-length EGFR) was used to initially identify cases; this finding was not reproduced in another study that sequenced the full-length EGFR cDNA (93). It will be important to resolve whether EGFRvIII is expressed with any appreciable frequency in HNSCC, as EGFR kinase inhibitors have demonstrated clinical activity against tumors expressing this variant (94). Mutation or amplification of the *MET* (*c-Met*) RTK gene has been reported in some HNSCC cases (95). This finding is of clinical interest both because *MET* amplification is thought to confer resistance to EGFR-directed therapy (96) and because the small molecule therapeutic crizotinib, which inhibits both the MET and ALK kinases, has recently been FDA approved for use in lung cancers harboring *ALK* translocations (97).

While each of the above genes and pathways are associated with activities that may indirectly prevent programmed cell death, several constituents of the apoptotic signaling cascade may also have an important role in HNSCC. These include caspase-8 (*CASP8*), encoding the critical proapoptotic enzyme that initiates a cascade of proteolysis responsible for executing apoptosis and found to be mutated in 8% of samples in one exome sequencing study (11, 98, 99); and *BCL2*, encoding a key antiapoptotic regulator reported to be overexpressed in a fraction of HNSCC cell lines, particularly those with reduced expression of p63 (63).

Adhesion and invasion signaling through TGF- β /SMAD and FAT1

Inactivation of TGF- β signaling components is well established in human cancer, including HNSCC, most commonly through loss of TGF- β receptor (*TGFBR2*) and SMAD genes as a result of chromosome 18q deletion (100). Notably, although missense mutations in *TGFBR2* have been previously described in primary HNSCCs (101), and *SMAD2* and *SMAD4* mutations have been reported in HNSCC cell lines (102), no point mutations in these genes were found through exome sequencing, perhaps due to the low frequency of these events. The TGF- β pathway is a pleiotropic regulator in human cancer, as mutational inactivation of its signaling components is associated with tumor initiation, while activation of the pathway is known to promote metastasis. Thus, genetic loss of TGF- β pathway factors would at first glance seem at odds with a contribution of this pathway to invasion and metastasis in HNSCC. Recent mouse models, however, suggest a more complex interaction. Conditional deletion of *Smad4* in the mouse stratified epithelium led to HNSCC in association with increased genomic instability and increased inflammation, the latter attributed in part to elevation of TGF- β 1 and activation of other SMADs in stroma, mucosal epithelia, and tumor cells (103). In addition, *Tgfb2* deletion within the mouse head and neck epithelia is insufficient to cause HNSCC, but cooperates with activated *Kras* to promote squamous carcinomas that metastasize to local lymph nodes (104). TGF- β 1 itself has also been associated with epithelial-



mesenchymal transition (EMT) and metastasis, the latter in the absence of functional TGF- β RII (105).

New insight into potential mechanisms of HNSCC invasion and metastasis was provided by the identification of mutations in the *FAT1* gene in nine HNSCC samples (12%) in one of the two exome sequencing studies (11). Six nonsense mutations and a seventh frameshift were noted, suggesting *FAT1* may function as a tumor suppressor. Notably, focal, intragenic homozygous deletions of *FAT1* have previously been described in oral cancer (106). As it is a member of the cadherin superfamily of cell membrane proteins that have demonstrated roles in the establishment of cell polarity and mediate cell-cell contacts, loss of *FAT1* might be predicted to permit loosening of the adhesions that normally restrain growth and/or migration of cells in an epithelial sheet. Similarly, mutations in genes encoding other membrane-associated proteins with a role in the establishment of polarity and cell adhesion have been described in HNSCC (107).

Additional genes/pathways

Several genes with unclear roles in HNSCC were found to be mutated at appreciable frequencies in at least one of the exome sequencing studies. Although the functional significance of the identified missense mutations is not clear (and some may merely represent passenger mutations), recurrent inactivating mutations were observed in several additional genes, suggestive of tumor suppressor activity. These include *MLL2* (11) and *NSD1* (11), both encoding histone methyltransferases, and *SYNE1* (10, 11), a nuclear envelope protein. Mutations within *MLL2* have recently been described in non-Hodgkin's lymphoma, and mutations within several other histone-modifying enzymes have been identified in renal cell carcinoma and diverse human cancers, suggesting a role for chromatin-mediated gene expression deregulation in cancer pathogenesis (108–110). Although *SYNE1* loss has been previously described in ovarian cancers and gliomas, this genomic locus spans more than 0.5 Mb (the longest isoform comprises 146 exons) and is subject to copy number variation in normal tissues. As such, this locus could be expected to accumulate relatively frequent passenger mutations, resulting in an overestimate of the significance of mutations if gene size is not taken into account (111–113).

Implications for therapy

The majority of patients with HNSCC come to clinical attention before the development of metastases, and as a result they have the potential to be cured with aggressive multimodality therapy (e.g., surgery, radiation, and/or chemotherapy). However, although improvements in surgical techniques, chemotherapy and radiation delivery, and supportive care have improved quality of life for patients with HNSCC, survival as a whole has not markedly improved (9).

As noted above, activating mutations in tyrosine kinases are relatively rare in HNSCC, and thus only one kinase-targeted therapy, cetuximab, is FDA approved for patients with HNSCC. This agent has generally modest efficacy, and efforts to identify a subset of patients whose tumors are “addicted” to EGFR signaling have not been successful (86, 87). One intriguing possibility is that production of EGFR activating ligands by tumor cells or tumor-associated stroma may increase sensitivity to EGFR inhibitors in HNSCC (114).

The recognition of HPV-related OPSCC as a distinct clinical entity with a better prognosis has led to the hypothesis that less-intensive therapy might mitigate toxicity without affecting survival. Clinical trials to test this hypothesis are now underway (115), but it should be weighed against the possibility that the primary survival benefit

of intensive therapy may lie with HPV-positive patients (116). Also relevant is the demonstration that human cancer cell lines derived from HPV-positive HNSCCs may be addicted to the continuous activity of the viral E6 and E7 proteins, suggesting that the latter represent important therapeutic targets. In addition, endogenous expression of E6 and E7 depends in part on the activity of transcription factors (including Notch), which themselves are controlled by potentially targetable upstream signaling pathways (52, 53).

The convergence of many HNSCC cancer genes on the activity of the G₁ cyclin-dependent kinases (e.g., CDK2, -4, and -6) suggests that targeting these kinases could have potent efficacy. A number of direct CDK inhibitors are currently being evaluated in clinical trials; several appear particularly effective in combination with cytotoxic agents with established efficacy in HNSCC (e.g., cisplatin, taxol; reviewed in ref. 34). A potentially novel approach to targeting the cell cycle in HNSCC has been suggested by the finding that inhibitors of the deubiquitinating enzyme USP2 could promote the proteasomal degradation of cyclin D1 (117, 118).

The ability to selectively target tumors with decreased p53 activity could have major implications for the majority of HNSCC patients. Unfortunately, the majority of p53-selective compounds currently in early phase clinical trials are designed to induce the activation of wild-type p53 in cells in which p53 activity is down-modulated by alterations in endogenous p53 regulators. These agents are unlikely to be effective in tumors in which p53 activity is abrogated primarily through mutation. An exception could be the large class of structural mutations that affect p53 protein stability; in these cases, decreasing p53 degradation could lead to restoration of some p53 activity (16). In addition, a variety of creative preclinical strategies have been undertaken to target tumor cells expressing mutated p53. These include the development of compounds that stabilize p53 through allosteric binding and synthetic lethal screens to identify candidate agents (including polo-like kinase inhibitors, some of which are in clinical trials) that selectively induce toxicity in p53-deficient cells (119).

Conclusions

As noted above, the relative lack of readily targeted oncogenes in these tumors has challenged traditional approaches to drug development for HNSCC. Nevertheless, the increasingly refined knowledge of epithelial biology and HNSCC genetics, combined with newer approaches aimed at targeting proteins previously thought undruggable, could lead to novel therapies with greater potency and less toxicity for these cancers (120). These conceptual advances will allow the development of ever-more-sophisticated animal models, which in turn will be essential for testing the emerging host of candidate targeted therapeutics, and for determining which of these may have the most clinical viability. A strong biologic rationale will be essential if progress against this disease is to be accelerated in the near term.

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