New advances in molecular approaches to head and neck squamous cell carcinoma

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Head and neck squamous cell cancer is the sixth most common cancer in the world. Despite advances in combined modality therapy, poor outcomes continue to be observed in the form of locoregional recurrence, metastasis, and development of second primary tumors. As tumors vary in their molecular and genetic etiology and because often there is already deregulation at the molecular level in otherwise histopathologically normal tissue, risk stratification using clinical and pathologic criteria alone has proved to be inadequate. In this article, the reader will gain an appreciation for the current advances in biomarker discovery using advanced technology and data interpretation in microarray analysis and proteomics. In addition, other molecular targets, aside from epidermal growth factor receptor, are discussed in the context of their promising role in predicting recurrence, response to therapy, survival, and overall prognosis. *Anti-Cancer Drugs* 22:656–664 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Head and neck squamous cell cancer (HNSCC) is the sixth most common cancer in the world with over 560 000 new cases diagnosed annually and over 300 000 deaths per year [1]. Surgery, chemotherapy, and radiation remain the mainstay of patient care. Despite advances in combined modality therapy, poor outcomes continue to be observed in the form of locoregional recurrence, metastasis, and development of second primary tumors, with the 5-year overall survival rate at less than 50%. As the molecular pathways involved in the development and progression of HNSCC become elucidated, it is clear that HNSCC is a heterogeneous group of cancers, stemming from varying molecular and genetic alterations. The diagnosis and prognosis of HNSCC is largely based on clinicohisto-pathology classification, mainly tumor, node, metastasis staging through clinical and radiographic assessment, surgical margin status, and histologic morphology and differentiation. As tumors vary in their molecular and genetic etiology and because often there is already deregulation at the molecular level in otherwise histo-pathologically normal tissue, risk stratification using clinical and pathologic criteria alone has proved to be inadequate. The emerging role of molecular biomarkers in detection, diagnosis, response to treatment, and prognosis has the potential for more accurate tumor staging and to facilitate management of HNSCC subtypes.

A biomarker is any objectively measured characteristic that may provide an indication of an outcome in question (i.e. response to treatment, metastasis, survival, etc.) [2].

Human papilloma virus (HPV) and epidermal growth factor receptor (EGFR) are currently the two most well studied and frequently used biomarkers in HNSCC. Several other potential targets have also been studied and show promise in predicting disease course. In addition, advanced technology and data interpretation in microarray analysis and proteomics have led to the identification of molecular genetic signatures with biomarker potential in predicting recurrence, metastasis, response to therapy, and survival.

Microarray analysis in head and neck squamous cell carcinoma prognosis

Gene expression profiling of primary tumors using microarray technology has played a significant role in enhancing our understanding of HNSCC molecular pathogenesis. Microarray analysis is useful in distinguishing gene expression differences among normal and malignant tissue, and many individual genes involved in the oncogenic process have been discovered and studied through this method. Microarrays have been used in both the study of known genes and the discovery of new genes. However, once a screen for molecular markers of disease by identifying differentially expressed genes between normal and malignant tissue, microarray analysis has more recently evolved into the characterization of gene sets that identify subgroups of HNSCC. These unique molecular signatures are currently being studied as indicators and predictors of disease and disease progression, response to therapy, and survival (Table 1).

Table 1   Microarray studies in HNSCC

Pathway	Genes	Prognosis	References
ECM remodeling	*FAP, PLAU, COL5A1, COL5A3, LAMB1, MSC, LLGL2*	Metastasis	Hensen *et al.* [3]
Focal adhesion, ECM remodeling	*ITGB4, LAMA3, COL3A1, 4A1, 5A1, 5A2, 11A1, FN1, PDGFC, TNC ITGA3, ITGA4, ITGA6, ITGB6, LAMA3, LAMB3, LAMC2, COL4A6, PGF, VEGFC,* and *MET*	Poor survival	Thurlow *et al.* [4]
ECM remodeling, MMP regulation	*SHC1, GRB2, SOS1, HRAS, RAF1, HRAS, RAC1, PAK1, PAK2, MAP3K4*	Metastasis	Hensen *et al.* [3]
Cell adhesion, motility	*ADAM9, ARHGAP5, ICAM3, LAMA3, MYH10, MYO5A, TNC,* and *TSPAN7*	Important in response to therapy	Dumur *et al.* [5]
Cell adhesion	*IL8, JUP, TGFBI, ATP2A2, LOXL2*	High-risk, decreased recurrence free survival	Chung *et al.* [6]
Epithelial-to-mesenchymal transition	*INHBA, HIF1A, PROCR, MMP2, PDGFRA*	High-risk, decreased recurrence free survival	Chung *et al.* [6]
NF-κB signaling activation	*IL8, SERPINE1, KRT14, BCL2A1, MMP1*	High-risk, decreased recurrence free survival	Chung *et al.* [6]
Hypoxia induced invasion	*HIF1α, MMP2, MMP13, PLAUR, P4HA1*	Metastasis	Hensen *et al.* [3]
Hypoxia-induced angiogenesis	*HGF, FGF2, PDGFβ, IL8, PGF*	Metastasis	Hensen *et al.* [3]
Cell proliferation	*TNC, PTHLH, CDC37L1, PCTK2,* and *SEPT11*	Increased in nonresponders to CRT/RT	Dumur *et al.* [5]
Apoptosis	*TOSO, BAG5, FOSL2, SULF1,* and *LTB*	Anti-apoptotic pattern increased in non-responders to CRT/RT	Dumur *et al.* [5]
Integrin signaling	*TSPAN7*	Increased in nonresponders to CRT/RT	Dumur *et al.* [5]
DNA mismatch repair pathway	*ANKRD17*	Increased in nonresponders to CRT/RT	Dumur *et al.* [5]
V2CR – innate immune response	*CCL3, CSF2, CXCL6, IL6, IL8, PDGFC, TGFB2, VEGF*	Poor survival	Thurlow *et al.* [4]
V3CR – adaptive immune response	*BLR1, CCL19, CCL21, CCL5, CCR2, CCR5, CCR6, CCR7, CXCR4, IL7, LTB, TNFSF4*	Good survival	Thurlow *et al.* [4]

CRT, chemoradiation; ECM, extracellular matrix; HNSCC, head and neck squamous cell cancer; MMP, matrix metalloproteinase; NF-kB, nuclear factor-κB; RT, radiation therapy.

Microarray chips permit the study of tens of thousands of genes at once, making this an efficient strategy to characterize HNSCC molecular signatures. Gene-based analysis is one method that can be supervised or unsupervised. Supervised gene-based studies use microarray analysis of predetermined genes of interest to determine differences in expression between two groups. Unsupervised analysis is used to screen for differentially expressed genes between two groups from a large pool of complementary DNAs. Gene-based analysis has been used to assess differences in expression of individual genes, whereas pathway-based analysis, in which functionally related sets of genes are studied, allows examination of differential gene expression in their biological context. Identifying any single common pathway from generated gene lists is challenging as selected genes are usually involved in multiple cellular processes. As generated gene lists do not explicitly expose any one molecular pathway, Gene Set Enrichment Analysis is used to interrogate expression data to determine the predominant biological processes at work.

One study used microarray analysis to generate a 75-gene list and then applied Gene Set Enrichment Analysis to identify enriched gene sets involved in molecular pathways that differed between high-risk and low-risk groups. High-risk tumors were those that had statistically significant decreased recurrence-free survival. The genes sets involved in epithelial-to-mesenchymal transition (EMT), nuclear factor-κB (NF-κB) signaling activation, and cell adhesion were found to be most significant in the high-risk group. EMT and NF-κB gene sets, specifically, were highly predictive of recurrence-free survival, suggesting that they could be used as a reliable prognostic biomarker of disease recurrence in HNSCC. Of note, gene sets prominent in the low-risk group were involved in mRNA splicing, cell cycle regulation, and mRNA processing [6].

Another group, using a reference dataset in addition to their own dataset of 11 metastasized and 11 nonmetastasized HNSCC tumors, validated gene sets that are associated with the propensity for tumors to metastasize. Supervised pathway-based analysis showed differential expression of genes involved in the extracellular matrix remodeling and the hypoxia-induced angiogenesis and invasion pathways between the two groups [3]. This study also highlighted that results from different microarray studies can be more readily compared if a supervised, pathway-based analysis is used. Results from gene expression studies using different microarray platforms can still be compared with this method as it draws on the biological functionality of gene groups, rather than solely individual genes themselves.

More recently, investigators used a novel method in which they applied spectral clustering and gene ontology through the Database for Annotation, Visualization and Integrated Discovery to microarray data to identify gene sets prognostic of HNSCC survival. Spectral clustering analysis of a microarray data set generates multiple vectors that embody the different dimensions within a given set and highlights heterogeneous gene expression aspects among samples. Each vector is the result of simultaneous reordering of the gene and sample data matrix in which the most important genes are found at the top and bottom of the vector gene list, and each vector pair (gene + sample) represents different existing elements of variance. Spectral clustering is an unsupervised analysis of microarray data that avoids bias inherent in supervised pathway-based analysis. Further, applying gene ontology to the spectral clustering-generated vector gene lists identifies enriched gene sets that are representative of major biologic pathways. Thus, the combined approach of applying spectral clustering and gene ontology to microarray data allows for an unbiased approach to elucidating important pathways implicated in HNSCC.

Thurlow *et al.* [4] performed microarray analysis on 14 normal epithelium and 71 HNSCC samples from patients whose outcomes were known. Two important vectors that encompassed the most variance among the dataset were identified through spectral analysis, V2 and V3. V2 separated normal from HNSCC samples, while V3 underscored the heterogenic nature of the HNSCC group. Gene ontology applied to spectral clustering-generated vectors showed that the gene lists primarily composed of genes involved in focal adhesion (FA) and extracellular matrix remodeling, and cytokine–cytokine receptor (CR) pathways. Gene ontology applied to V2 and V3 specifically found that V2CR genes were related to innate immune response, whereas V3CR genes were involved in the adaptive response. Cox survival analysis used to determine the prognostic significance of these gene sets found the FA and CR gene sets to be significantly prognostic of patient survival in HNSCC. The FA gene set predicted poor outcome, whereas the adaptive immune response CR gene set (V3CR) predicted good outcome, which was not dependent on HPV status. Interestingly, the adaptive immune response gene set was a better prognostic indicator of favorable outcome in the oropharyngeal samples of this cohort compared with HPV markers. Aside from FA and CR gene sets, the relationship between the adaptive and innate immune response in determining outcome seems to be important in that a high ratio of V3CR/V2CR expression was found to be independently prognostic of survival. These findings were also confirmed in an independent data set [7]. In addition, of interest, the FA and CR prognostic gene sets outperformed current clinical and histopathological parameters in predicting survival outcome.

Other investigators used gene sets to predict response to chemoradiation (CRT) and radiation therapy. They identified 167 probe sets that differed in gene expression between complete responders and nonresponders to CRT or radiation therapy in 14 HNSCC patients undergoing therapy with curative intent. One hundred and forty-two of these probe sets, when further subjected to six different prediction algorithms, showed 93–100% prediction accuracy of response to therapy. Genes involved in cell adhesion and motility, regulation of transcription, cell proliferation, and apoptosis were found to be differentially expressed in complete responders and nonresponders to therapy. Of note, the investigators found the ankyrin repeat domain 17 gene, which plays a role in DNA mismatch repair pathways, to be upregulated in nonresponder tumor samples. These results show potential in the use of microarray analysis and genetic expression profiling as markers of prognosis of response to CRT and radiation therapy [5].

Together these studies show the emerging role of gene expression profiling in the development of unique HNSCC signatures to use as biomarkers in the predictors of response to therapy and in the prognostication of disease-free survival and overall survival. Genes involved in alterations of cell–cell adhesion, cell motility, extracellular remodeling, and EMT highlight the importance of microenvironment changes in prognosis, as they contribute to tumor recurrence, metastasis, and thus, poor response to therapy and overall survival. Pathways involved in EMT, such as loss of cell–cell adhesion and cell motility, are the same as those central to the metastatic process. Interestingly, NF-κB is one of the molecules involved in EMT-related cellular occurrences, showing the concerted orchestration in the development of aggressive cancers. In addition, the immune responses and the hypoxia-angiogenesis pathways are crucial in their effects on tumor growth, recurrence, and metastasis.

Genetic microarray analysis has the potential to study the expression of tens of thousands of genes at once in a cell or tissue sample. Its use in the clinical setting has already emerged in breast cancer diagnostics and management. The Oncotype DX test analyzes the expression pattern of 21 different genes specific to breast cancer to calculate a Recurrence Score, which characterizes the tumor as low, intermediate, or high risk. This score is used to estimate the risk of recurrence within 10 years from initial diagnosis and to tailor individual treatment accordingly. In HNSCC, validated correlation to known outcomes may allow the identification of definitive genetic profiles, which can be used in a similar manner as the Oncotype DX test, to both accurately diagnose and predict the prognosis and response to therapy of new malignancies.

Microarray analysis evaluates differences in gene expression, mainly mRNA transcription. However, the functional outcome of gene expression, in the form of the

posttranslationally modified protein, is not distinctly evaluated. The number of mRNA transcripts does not always correlate with protein expression and quantity. In addition, microarray analysis must become standardized so that studies can be compared and large datasets can be generated and validated. Although this technology remains promising, gene patterns and molecular signatures need to be further studied and validated in larger cohorts to be applied in clinical practice.

Serum and tissue proteomics

Proteomics accounts for posttranscriptional and translational modifications, and may be more accurate in reflecting the functional biological state of tumors, and thus perhaps a better biomarker discovery tool, than genomic analysis alone. A few methods, such as (i) two-dimensional differential in gel electrophoresis (2-D DIGE), which is limited by its poor resolution of low-molecular weight proteins; (ii) surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), which is based on ionization characteristics and mass-to-charge ratio; and (iii) matrix-assisted laser desorption ionization time-of-flight mass spectrometry, which is based on molar quantity, have been used alone or in combination to date. Proteomic analysis by SELDI-TOF-MS has been shown to differentiate, with high sensitivity and specificity, both HNSCC tumors from normal tissue and pretreated and posttreated HNSCC [8–11]. Research in the use of proteomics in both detection, for diagnosis and screening purposes, and posttreatment surveillance has recently been of great interest (Table 2).

In one prospective study, serum samples collected from 143 patients with HNSCC were analyzed by 2-D DIGE in combination with matrix-assisted laser desorption ionization time-of-flight mass spectrometry and correlated with disease status. Recurrent HNSCC was significantly associated with underexpression of kininogen and serine protease inhibitors C-1 inhibitor, serine/cysteine proteinase inhibitor clade G member 1, and kininogen and angiotensinogen, and overexpression of thiol-specific antioxidant proteins, apolipoprotein A1, proapolipoprotein, and epidermal cytokeratin 2. These protein expression differences in protease inhibitors, kininogen, thiol-specific antioxidant proteins, and apolipoproteins are prospective markers of recurrence that have the potential to identify those tumors that are more aggressive and also have the potential to be used in disease surveillance [12].

In another study, 2-D DIGE analysis was used to examine protein expression patterns in HNSCC and matched normal tissue. Proteomic characterization showed significant differences in 17 proteins, four of which have not been studied earlier. Among the novel proteins, glutathione synthetase was found to be upregulated, whereas cornulin, guanylate binding protein 6, and heat

Table 2 Proteomic studies in head and neck squamous cell cancer

Protein profile	Significance	References
Underexpression: kininogen and serine protease inhibitors C-1 inhibitor, kininogen, angiotensinogen, serine/cysteine proteinase inhibitor clade G member 1	Recurrence	Gourin et al. [12]
Overexpression: thiol-specific antioxidant proteins, apolipoprotein A1 and proapolipoprotein, epidermal cytokeratin 2	Recurrence	Gourin et al. [12]
Upregulated: keratin 17, Mn superoxide dismutase, proliferating cell nuclear antigen, glutathione synthetase, ferritin light chain, keratin 5	Increased in HNSCC vs. matched normal	Merkley et al. [13]
Downregulated: cornulin, keratin 4, cystatin-B, cytokeratin 13, leukocyte elastase inhibitor (Serpin B1), Annexin A1, cytokeratin 8, guanylate binding protein 6	Decreased in HNSCC vs. matched normal	Merkley et al. [13]
Overexpression: stratifin (14-3-3sigma), YWHAZ (14-3-3zeta), S100-A7	3 molecule biomarkers to identify HNSCC	Ralhan et al. [14]
Cystatin A, S100A8, and 4 un-identified proteins	Marker to predict recurrence/ progression free survival	Roesch-Ely et al. [15]
Vimentin, prohibitin, EVI-5, cathepsin D	Expressed in HNSCC, potential biomarkers	Patel et al. [16]

HNSCC, head and neck squamous cell cancer.

shock 70 kDa protein 5 were downregulated. Cornulin was the highest-ranked differentially expressed protein and loss of expression was validated in an independent cohort. Cornulin, otherwise known as squamous epithelial heat shock protein 53 and a member of the calcium binding S-100 family, is involved in the stress response of squamous epithelia. Reduced expression may make squamous epithelia more vulnerable to repeated chemical and infectious offenses that eventually lead to carcinogenesis. Other notable differences in protein expression included the upregulated keratin-17, which has been associated with metastasis [17], and proliferating cell nuclear antigen, which has been associated with poor prognosis in other studies [18]. Other markers reported earlier and confirmed to be downregulated in HNSCC compared with normal tissue in this study included cytokeratins 4, 8, and 13; keratin 4; annexin A1; transglutaminase 3; cystatin B; and leukocyte elastase inhibitor (Serpin B1) [13]. Using 2-D DIGE, the researchers were able to identify abnormal underexpression and overexpression of structural and cell proliferation proteins and those involved in the oxidative stress responses in HNSCC.

Proteomic analysis of normal epithelia and well-differentiated, moderately, and poorly differentiated HNSCC has showed significant differences at each stage that may provide insight into the molecular pathology of HNSCC progression. In one study, the relative expression of proteins in expected cellular functions and pathways, such as structural, signal transduction and cell cycle

regulation, and extracellular matrix degradation and migration, had a unique pattern with histopathological correlation. Vimentin, which is involved in EMT, was the most unique to HNSCC samples. The well-differentiated tumors differentially expressed cytoskeletal and Notch pathway proteins, such as delta 4 and delta 1, while the moderately differentiated groups expressed placental cadherin and protocadherin γ A6. In contrast, poorly differentiated tumors expressed AF1q, a potential oncoprotein, and other proteins involved in fatty acid metabolism and membrane trafficking. The stem cell protein PIWIL3 was found in only normal and well-differentiated tumors; oncoprotein DJ-1 was found in both well-differentiated and moderately differentiated tumors; and JWA, a protein involved in cell migration, and signal transducer and activator of trancription 3 (STAT3) along with its activator, Janus-activated kinase 2, were found in both moderately differentiated and poorly differentiated tumors.

Prohibitin, which plays a role in Ras activation of the Raf/ MEK/ERK pathway [19], cell adhesion, and cell migration, and EVI-5 were found only in tumor samples. Prohibitin and EVI-5 likely contribute to deregulated cell proliferation in HNSCC and are candidates for further studies as to their use of biomarker potential. Of note, the prominent protease in tumor samples was cathepsin D, which has been studied in breast cancer in which its expression levels correlate with metastasis and poor survival [20]. HSP27, HSP70, glutathione-S-transferase, and integrin β4 were other proteins that were overexpressed in malignancies compared with normal controls [16].

Evaluation of histologically normal mucosa in HNSCC may be able to predict the development of second primary tumors and local recurrence. A prospective pilot study used SELDI-TOF-MS analysis protein profiling of biopsies from five sites between the oropharynx and esophagous in 24 patients with HNSCC. The highest-ranking proteins, differentiating normal from HNSCC mucosa, included cystatin A, followed by peaks with m/z 10464 and m/z 12337 (both unidentified), S100-A8, and by two other unidentified peaks with m/z values of 12910 and 3640. Together, these six protein peaks were able to identify those mucosal sites more prone to carcinoma development at the molecular level, even if they appeared to be histologically benign. The researchers were able to predict tumor recurrence and progression-free survival using these protein profiles. This study suggests that proteomic analysis of additional biopsies taken from other regional sites at the time of initial tumor workup could allow for individualized HNSCC prognostication in the future [15].

Another study used multidimensional liquid chromatography and tandem mass spectrometry to analyze isobaric mass tag-labeled HNSCC and control samples to detect differences in protein expression. Structural proteins, in addition to signaling proteins and transcription factors, were found to be among those consistently differentially expressed in HNSCC. Overexpressed YWHAZ, stratifin, and S100-A7 were the most important in discrimination, and when used in combination had a sensitivity of 92% and a specificity of 91% in distinguishing HNSCC from normal tissue. This finding was validated in an independent cohort, which had a sensitivity of 92% and a specificity of 87%. These three proteins merit further investigation into their usefulness as biomarkers for screening and diagnosis in HNSCC [14].

These cumulative findings suggest that proteins involved in signal transduction and proliferation, and oxidative stress responses, are important in the development of HNSCC. In addition, deregulation in proteins involved in cell structure, adhesion, migration, and extracellular matrix remodeling are associated with increased recurrence, metastasis, and poor survival. Identification of differential protein patterns, similar to genomic signatures, has the potential to stratify patients according to the aggressiveness of their disease.

Molecular targets: epidermal growth factor receptor and beyond

EGFR has been studied extensively as a therapeutic target and a prognostic biomarker in HNSCC. EGFR is a transmembrane tyrosine kinase receptor that is overexpressed, early in HNSCC carcinogenesis [21]. EGFR ligand binding causes homodimerization or heterodimerization, with other members of the human EGFR family including ErbB2, resulting in autophosphorylation and activation of multiple downstream signaling pathways; most notably the PI3K/Akt, Ras/Raf/MEK/ERK1/2, and STAT pathways. Aberrations in EGFR and these signaling pathways lead to uncontrolled cell proliferation, contributing to cancer development and progression.

More than 90% of all HNSCCs overexpress EGFR in which increased levels are associated with poor prognosis in the form of high locoregional failure and decreased survival [22–24]. In 2002, Ang *et al.* [23] found that the overall survival and disease-free survival were significantly lower and that locoregional recurrence was significantly higher in patients that harbored tumors with high EGFR expression when compared with those with low EGFR expression. Interestingly, however, EGFR levels did not correlate with distant metastasis. EGFR gene amplification, as detected using fluorescence in-situ hybridization, is one potential mechanism of overexpression in which increased EGFR copy numbers has been associated with worse progression-free survival and overall survival [25].

Owing to its central role in HNSCC carcinogenesis, several methods of targeted therapy toward EGFR have been developed. Broadly, these therapies include monoclonal antibodies, tyrosine kinase inhibitors, antisense

oligonucleotides, and small interfering RNAs that respectively aim at attacking EGFR in both its extracellular and intracellular domain and at the translational stage. At present, cetuximab, an EGFR monoclonal antibody, is the only approved EGFR targeting drug for use. Cetuximab in conjunction with radiation therapy has been shown to be effective in locoregionally advanced HNSCC [26]. Cetuximab also showed a survival benefit in the EXTREME study when used as a first-line agent in combination with platinum-based computed tomography in recurrent and metastatic HNSCC [27].

Despite repeated evidence that EGFR overexpression is a poor prognostic factor in HNSCC, neither the levels of EGFR nor EGFR gene amplification has been consistently associated with clinical response to EGFR targeting. The only exceptions were a phase III cetuximab and platinum trial in which patients with low EGFR expression had a marginally better response to cetuximab [28] and a phase I EGFR antisense gene therapy study in which patients whose tumors expressed higher levels of EGFR were most likely to respond to treatment [29].

More recently, the application of EGFR expression status to predict the occurrence of a second primary lesion has been examined. A study analyzing EGFR expression in primary laryngeal HNSCC found significant positive correlation between EGFR expression and the development of subsequent primary tumors [30]. The phosphorylation and mutational status of EGFR in HNSCC prognosis was also recently investigated in a prospective study. EGFR sequencing and western analysis to determine EGFR expression and hyperphosphorylation were performed on malignant tissue from 82 consecutive patients that had not been treated earlier with EGFR-targeted therapy. EGFR hyperphosphorylation was found to be associated with increased number of lymph node metastases and higher nodal stage of disease. Even after accounting for overexpression, tumors with hyperphosphorylated EGFR relapsed significantly earlier compared with those without [31]. To date, EGFR phosphorylation status has not been well studied as a marker of response to EGFR-targeted therapy. Although EGFR expression appears to be a consistent marker of poor prognosis, its utility in predicting response to EGFR-targeted therapies remains incompletely understood.

Other potential molecular targets
In addition to EGFR, STAT proteins play a major role in the signaling pathways involved in HNSCC, among other carcinomas. STAT3, in particular, is overexpressed or constitutively activated in many HNSCCs [32]. Tyrosine phosphorylation of STAT3 in the cytoplasm results in homodimer or heterodimer formation, followed by translocation to the nucleus in which STAT3 dimers activate antiapoptotic and proproliferative genes. Earlier studies have reported that pSTAT3 expression in the primary HNSCC tumor is associated with reduced survival, alone or in the context of expression of coordinate signaling pathways [33,34]. A recent study showed that nuclear localization of STAT3 in HNSCC tumors was associated with a favorable prognosis. High expression of STAT3 in the nucleus versus the cytoplasm was found to be associated with longer progression-free survival and lower risk of death in two independent cohorts [35]. STAT3 has also been studied in the context of molecular-targeted therapy. Both in-vitro and in-vivo studies show the antitumor effects of STAT3 targeting using oligonucleotide decoys [36,37], which bind to STAT3, thereby preventing its binding to promoter sequences and activation of proproliferative genes, in the treatment of HNSCC. However, the main obstacle in the development of the STAT3 decoy remains the limited ability to withstand serum endonucleases and exonucleases, which currently prevent systemic administration. Nevertheless, STAT3 shows great potential in its use as both a biomarker for disease stratification and a molecule for targeted therapy development.

Hypermethylation of E-cadherin may also be an independent marker of improved HNSCC survival. Promoter hypermethylation of the cadherin type 1 gene, which encodes E-cadherin, inhibits E-cadherin expression. Although loss of E-cadherin has been associated with aggressive HNSCC disease, hypermethylation of cadherin type 1 gene has significantly better overall survival when compared with tumors without hypermethylation, even after controlling for HPV status, age, sex, and stage [38].

Several other groups have studied the role of a variety of molecules associated with carcinogenesis in predicting recurrence, decreased response to therapy, and poor overall survival and prognosis in HNSCC (Table 3). One relatively large ($N = 444$) longitudinal, prospective cohort study, which controlled for age, sex, smoking, stage, site of origin, and comorbidities, found increased pretreatment serum interleukin-6 (IL-6) levels to be an independent predictor of both recurrence and poor survival. Thus, pretreatment IL-6 levels could be a biomarker for earlier detection of relapse [39]. Serum cytokine and angiogenic factor (CAF) profiling also seems to be useful in determining recurrence. In a phase II induction therapy trial of carboplatin, paclitaxel, and cetuximab, baseline and posttreatment levels of several tumor hypoxia-regulated CAFs were related to patient outcome. Eight CAFs in particular were categorized as 'high risk'. These included vascular endothelial growth factor, IL-4, IL-8, osteopontin, growth-related oncogene-α, eotaxin, granulocyte colony stimulating factor, and stromal cell-derived factor-1α. An increase in six or more of these high-risk CAFs was 73% specific and 100% sensitive for predicting disease progression and strongly associated with shorter time to relapse [40]. In a multivariate

Table 3 Molecular targets in head and neck squamous cell cancer

Marker	Prognosis	References
EGFR amplification and overexpression	Poor survival	Ang et al. [23]
Nuclear STAT3	Increased progression-free survival, lower risk of death	Pectasides et al. [35]
Hypermethylation of E-cadherin gene	Improved overall survival	Marsit et al. [38]
Increased IL-6	Recurrence and poor survival	Duffy et al. [39]
Cytokine and angiogenic factors: VEGF, IL-4, IL-8, osteopontin, growth-related oncogene-α, eotaxin, granulocyte colony stimulating factor, and stromal cell derived factor-1α	Recurrence and increased progression	Byers et al. [40]
Increased PTEN	Radioresistance, and worse locoregional control	Pattje et al. [41]
Decreased HLA class I antigen	Decreased disease-free survival	Bandoh et al. [42]
Downregulation of SERPINB13	Poor grade, lymph node metastasis, reduced survival	De Koning et al. [43]
ANO1 amplification and overexpression	Increased metastatic potential (in vitro)	Ayoub et al. [44]

EGFR, epidermal growth factor receptor; HLA, human leukocyte antigen; PTEN, phosphatase and tensin homolog; STAT3, signal transducer and activator of trancription 3; VEGF, vascular endothelial growth factor.

analysis examining 140 locally advanced HNSCCs that were surgically resected and radiated postoperatively, Pattje et al. [41] found phosphatase and tensin homolog deleted on chromosome 10 to be an independent predictor of worse locoregional control after accounting for lymph node metastases and extranodal spread. In-vitro studies by the same group also found that increased phosphatase and tensin homolog expression was related to radiation resistance. Others compared the expression of human leukocyte antigen class I antigen, transporter associated with antigen processing, and tapasin in primary and matched metastatic HNSSC lesions. These proteins are involved in antigen presentation and in eliciting the cytotoxic T cell immune response. Although downregulation occurred across this panel of proteins in both groups, the decrease was more significant in metastatic tissue. Decreased human leukocyte antigen class I antigen levels in metastatic samples were found to be independent markers of reduced progression-free survival and poor prognosis [42]. SERPINB13, which belongs to the serpin family of intracellular serine protease inhibitors, was examined in a cohort of 99 HNSCCs. It was found that SERPINB13 was either partially or completely downregulated in 75% of samples compared with normal epithelial tissue, confirming earlier findings of mRNA and protein downregulation of this molecule. When correlated to outcome, the investigators found that low protein levels of SERPINB13 were associated with poor tumor differentiation, lymph node metastasis, and decreased disease-free survival and overall survival. It seems that downregulation of SERPINB13 may be yet another

biomarker for reduced survival in HNSCC [43]. The role of ANO1, a calcium-activated chloride channel, has also been investigated in HNSCC. The investigators found that ANO1 gene amplification and overexpression are involved in cellular processes that increase metastatic potential, making ANO1 a novel target to prevent metastasis and improve patient survival in HNSCC [44].

Taken together, these studies are just a small sampling of the various molecules currently under investigation in their roles as biomarkers. They all share common features that make them attractive candidates of pursuit. Whether the above-mentioned molecules are overexpressed, underexpressed, or vary in their localization, it seems that differences exist in these parameters when HNSCC is compared with normal mucosa. Furthermore, these differences have been analyzed and statistically correlated to available clinical outcome data to elucidate significant relationships, which suggest a potential for each studied molecule to be a biomarker of some sort (i.e. recurrence, radiation therapy resistance, survival) in HNSCC. In addition, a number of these molecules have been studied in preclinical models (i.e. STAT3) in which targeting is associated with antitumor effects; showing promise in the development of future HNSCC-targeted therapies.

Conclusion

HNSCC is a heterogeneous disease despite the presence of common histologic features. Most patients present with advanced disease, and thus the disease-free survival and overall survival remain suboptimal. The overall 5-year survival rate has not changed significantly in the last 20 years, despite continued advances in the field. It is important to develop reliable methods of elucidating the specific features that are critical to tumor progression for each patient to achieve the promise of personalized medicine. EGFR overexpression is a poor prognostic marker, but to date has not consistently predicted response to EGFR targeted therapy. HPV has emerged as a distinct biological entity that portends favorable prognosis and response to therapy, regardless of the specific therapeutic regimen. Gene-discovery efforts including microarray analyses and advances in bioinformatics have allowed for recognition of differential gene expression. Elucidation of the different molecular signatures of HNSCC will help to unravel the distinct underlying mechanisms in this heterogeneous group of cancers, and provide a source of novel molecules for future research and the development of targeted therapies. In addition to microarrays, newer proteomic approaches are also under investigation. Ideally, the development of clinically useful methods to assess HNSCC tumors can be identified to allow individual patients with HNSCC to receive the therapy most likely to be effective for their individual tumor.

Acknowledgement

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