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Update in Molecular Diagnostic Tests in Head and Neck Cancer

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Abstract

Head and neck cancers (HNC) include a variety of cancers originating in the upper airways that represent a variety of histology. The most common type of HNC is squamous cell carcinoma (SCC), which is linked to tobacco and alcohol use and to human papilloma virus (HPV). At present, there are no standard molecular tests that are routinely used in the clinics. This overview will discuss the current knowledge on molecular markers with the potential to be developed as diagnostic tests for cancer risk assessment, early detection, clinical response prediction to specific therapies or prognosis. These markers are usually based on recent findings in tumor biology and genetic defects in HNC, and provide information both independently and in combination with currently available clinical parameters. In practice, many potential markers are difficult to measure due to assay variability, lack of standards for the interpretation of assay results and incomplete knowledge of the effects on disease biology and response to treatment. However, there is great enthusiasm for the general concept of using molecular knowledge for clinical management of HNC. Although it will be a great challenge to develop robust and reliable molecular diagnostic tests, the development of promising assays fueled by advances in science and technology will continue and ultimately reach the goal of improving the care of HNC patients.

I. Introduction

Head and neck cancers (HNC) mostly originate from the mucosal epithelia of the oral cavity, pharynx, and larynx. Due to the wide variation in tissues of origin, HNC is considered a heterogeneous disease, representing a variety of histology and differentiation patterns. The most common histology is squamous cell carcinoma (SCC), which comprises greater than 90% of HNC. Unlike breast cancer, where the molecular testing of hormone receptor protein expression and HER2 gene amplification is a standard and integral part of the clinical decision making process, there are no standard molecular tests in head and neck cancer. Current treatment protocols tend to call for dose-intense treatments through aggressive induction chemotherapy and concurrent chemoradiation, with an expanding role for targeted agents. There is growing need for clinically meaningful molecular tests to augment currently available clinical parameters for choosing treatment with maximum efficacy and minimal toxicity. In

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this report, we will present the data for emerging molecular tests and discuss the necessary validation steps for the tests to be the standard of care in the management of HNC.

II. Molecular tests for early detection of malignancy

1. Serum multiplexed cytokine and proteomic profiling

Cytokines and chemokines, small molecules secreted by cells of the immune system, modulate inflammatory responses in humans. Individually, a single cytokine can provide little information about the signaling cascade of inflammation, but the complex web of inflammatory markers may have clinical utility in the search for HNSCC biomarkers. It is known that aberrations of serum inflammatory and angiogenic markers occur frequently in HNSCC. Traditionally, ELISA has been used to assay for cytokine activity which allows analysis of one protein at a time. With the development of high throughput technologies such as the Luminex system, more accurate and sensitive assays for measuring serum cytokine levels are gaining acceptance.^{1,2} Recently, Linkov, *et al.* reported the result of multiplexed serum analyses including 60 cytokines, growth factors, and tumor markers in a cohort of 116 pre-treatment HNSCC patients, 103 successfully treated patients who had no evidence of disease for at least 3 years and 117 age, sex and smoking status matched controls. A panel of 25 biomarkers showed the highest diagnostic power with 92% accuracy to distinguish the cancer patients from the controls.²

Also, by analyzing serum from HNSCC patients in comparison with healthy controls using matrix-assisted laser desorption and ionization (MALDI) mass spectroscopy (MS) and surface enhanced laser desorption and ionization (SELDI) MS, HNSCC patients could be distinguished reliably in several studies.^{3–6} In the study by Sidransky, *et al.*, sera from 99 HNSCC patients and 143 control subjects were analyzed using MALDI-MS and the patients were predicted with a sensitivity of 70% and specificity of 90%.⁶ Despite the criticism for these types of descriptive studies for lack of reproducibility and biological explanation, these serum tests represent a promising new approach in early detection of HNC as well as a surveillance method for early detection of recurrence because of the ease of obtaining serum samples and the relatively low costs for high-throughput screening tests. As was shown by Taguchi *et al.*, a solid study design coupled with analysis on multiple MALDI-MS platforms can circumvent many of the initial shortcomings that characterized early studies using MALDI-MS.⁷

2. Genomic and proteomic profiling of saliva for the early detection of oral SCC

Genomic and proteomic technologies are also being applied to analyses of easily accessible saliva in an attempt to find early biomarkers of oral SCC. Using saliva as a diagnostic medium for oral SCC was initially pioneered by Li, *et al.*^{8,9} They have shown that RNA can be isolated from saliva to generate a salivary transcriptome and identified salivary mRNA levels of IL8, SAT and H3F3A as predictive markers of oral SCC with both sensitivity and specificity of 90%.⁹ Similarly, the saliva was analyzed using proteomic techniques and appears to be promising.¹⁰ Other studies have confirmed the general feasibility of proteomic techniques to study a subset of the salivary proteome¹¹, but larger studies using more sensitive techniques will need to be performed on well-documented patient populations to test whether this approach can identify biomarkers for the detection of early HNC.

III. Molecular tests for prognosis in HNC

1. Detection of human papilloma virus (HPV) as a favorable prognostic marker

For over a decade, the human papilloma virus (HPV) has been accepted as the causative agent in human cervical cancer, as well as most other anogenital malignancies.¹² Over 70 different genotypes of these small, non-enveloped, epitheliotropic DNA viruses have been identified to

date, but only a small percentage of these genotypes are linked to carcinogenesis. Genotypes 16, 18, 31, 33, 35, 39, 45, and 52 are considered “high risk” HPVs because of their association with carcinoma of the uterine cervix, while HPV 16 being linked to the majority of HPV-positive HNSCC.^{13–14} Approximately 20% of HNSCC are HPV positive, although the incidence is much higher in oropharyngeal SCCs where about half of the carcinomas test positive for HPV.^{15,16}

The HPV positive HNSCC population appears to be biologically and clinically distinct from the HPV negative patients. A patient with a HPV-positive cancer is less likely to be a smoker or consume alcohol.^{13,17,18} The median age of diagnosis is five years younger in HPV-positive patients. HPV infection is a sexually transmitted disease, and high risk sexual behavior has long been linked to the development of uterine cervix carcinoma. Similarly, high risk sexual behavior (in particular oral-genital contact), oral HPV infection and HPV 16 exposure have been epidemiologically linked to the subgroup of oropharyngeal HNSCC.¹⁸ In a large cohort study, serum antibodies against HPV 16, but not other HPV types was associated with a 2.2-fold increase in risk to develop HNSCC, even when other confounders such as smoking and age were taken into account.¹⁹ Since serum antibodies against HPV are an indirect and thus imperfect measure of HPV exposure, these results are strongly suggestive for a causative role of HPV in HNSCC.

Another indication for the causality of HPV in HNSCC is the tumorigenic properties of HPV oncoproteins in cell model systems. An important step in this process is the integration of the virus into the host genome as a hallmark of tumorigenesis. (Although it must be noted that one third of cervical cancers carries the episomal form of the virus). Two main viral oncogene products, E6 and E7, bind to and inactivate the tumor suppressors p53 and pRb, respectively, leading to transformation.²⁰ Expression of these HPV oncoproteins is localized to cancer cells, but not to the surrounding stroma. E6 and E7 activity leads to a distinct pattern of cell cycle marker expression, which is clinically useful.^{21,22} HPV positive tumors demonstrate the loss of pRb and cyclin D1 expression, overexpression of p16, and rarely have p53 mutations. On the other hand, HPV negative tumors overexpress pRb and cyclin D1, and display loss of p16 and frequent p53 mutations.^{23–24}

With the implementation of the Papanicolou (Pap) smear, the incidence of and mortality from cervical cancer have dropped dramatically over the past half century, and the efficacy of screening is clear. The Pap smear utilizes microscopic examination of exfoliated cells by a pathologist, and the sensitivity and specificity of this technique varies widely.²⁵ In one meta-analysis, the sensitivity ranged from 30–87%, with specificities in the 86 to 100% range.²⁶ HPV detection using PCR and hybridization methods has also been incorporated into routine screening, allowing for early detection and eradication of preinvasive cervical dysplasia. Similar techniques, i.e. oral Papanicolou smears (cytologic brush specimens) and FISH for chromosomal aberrations, have shown promise in one small study of patients with documented HNSCC.²⁷ However, the utility of pap smears in screening for tonsillar head and neck cancer has been suggested, but not proven.

In-situ hybridization (ISH) involves DNA probes, which are applied directly to the tissue and localize to the target sequence (Figure 1).¹⁸ To date, ISH for HPV is the only clinically useful test that is available, but only at tertiary referral centers. This technique is much less sensitive and more time consuming than PCR. Real-time PCR, when performed on microdissected tumors, can detect as few as 10–100 DNA molecules, and can isolate individual HPV types.²⁸ The advantage of ISH over PCR, however, is that it proves that the virus is present and active in the cells of interest while PCR merely demonstrates that HPV is present in the clinical specimen.

HPV-positive tumors demonstrate overexpression of p16, and this protein may serve as a surrogate marker for high risk HPV. Using immunohistochemistry, strong correlations have been found between diffuse nuclear and cytoplasmic staining of p16 and HPV DNA by ISH and real-time PCR.^{29,28} An HPV positive cervical lymph node in a patient with an occult primary tumor should lead the oncologist to look for a primary oropharyngeal SCC.³⁰

HPV-positive tonsillar cancers have a much better prognosis, including a 60–80% risk reduction of death, than their HPV negative counterparts.^{31–33} This may be due in part to the fact that tonsillar HPV positive tumors tend to occur in non-smokers and non-drinkers, where p53 mutations are uncommon, and there is assumed to be minimal field cancerization effect. With unmutated p53, an intact apoptotic response allows for a better response to chemoradiotherapy.³⁴ Currently, conducting clinical trials for HPV positive patients with decreased treatment intensity in a way of minimizing unnecessary toxicities are in active discussion by many investigators and cooperative trial groups.

Recently, a prophylactic vaccine composed of the HPV16 viral capsid protein, and a bivalent HPV 16/18 L1 virus-like particle vaccine, were shown to prevent the development of persistent infection, as well as the development of cervical dysplasia in two separate randomized trials.^{35,36} The role of these vaccines in oral cancers has not been evaluated, but given the link between high-risk sexual activity and HPV-positive HNSCCs, one would expect a benefit in this group of patients.

2. Genetic aberration of Epidermal Growth Factor Receptor as a poor prognostic marker

Epidermal growth factor receptor (EGFR), also known as ErbB1 and HER-1, is a member of the HER/ErbB family of receptor tyrosine kinases and is a 170kDa transmembrane glycoprotein which includes a tyrosine kinase enzyme within its intracellular domain. Transforming growth factor- α and amphiregulin are considered to be the two most important endogenous ligands for EGFR in HNSCC while epidermal growth factor, betacellulin and epiregulin play minor roles.^{37–38} EGFR has been implicated in the regulation of a variety of neoplastic processes, including, but not limited to, tumor invasion and metastasis, cell cycle progression, angiogenesis, tumor cell motility, and inhibition of apoptosis.^{39,40}

EGFR is overexpressed in a wide variety of carcinomas, including lung, head and neck squamous cell, colorectal, breast, renal cell, ovarian, and pancreatic carcinoma. The level of overexpression varies according to tumor type; in HNSCC, between 80 to 100 percent of tumors have increased EGFR protein levels.^{41,42} The prognostic and predictive values of EGFR overexpression have been widely studied. In most tumor types, EGFR overexpression correlates with a poorer prognosis and decreased overall survival.^{43–46}

A wide variety of assays are available for measuring EGFR, including fluorescent *in-situ* hybridization (FISH) and quantitative polymerase chain reaction (q-PCR) for gene copy numbers, reverse transcriptase polymerase chain reaction (RT-PCR) and DNA microarrays for RNA expression, and solid matrix blotting techniques, enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) for protein expression. Each technique has its advantages and disadvantages, and some have resulted in contradictory data. The most commonly used method of EGFR protein detection is IHC, and is relatively quick and easy to perform with readily available equipments in most laboratories. However, there is lack of standardization of IHC assays from various laboratories, inter- and intra-variability in scoring by pathologists and technical limitations due to the semi-quantitative nature and limited detection range of IHC that complicate the interpretation of protein expression results. However, these limitations are circumvented by using automated and more sensitive detection methods such as automated quantitative analysis (AQUA) using immunofluorescence.⁴⁷ In addition, although EGFR and other members of the *HER* family are considered to be

transmembrane tyrosine kinases, there is growing evidence that EGFR is able to undergo nuclear translocation, where it acts as a nuclear transcription factor.⁴⁸ Pathologists score EGFR expression based on membrane and cytosolic staining, overlooking the nuclear component. Through the use of AQUA, Psyrri, *et al.* showed that both tumor EGFR and nuclear EGFR staining were independent prognostic factors.⁴⁷

In FISH, a complementary labeled nucleotide probe is used to localize a specific DNA target sequence in tissue section (Figure 2).⁴⁹ This technique is routinely used to detect HER-2/neu amplification in breast cancer. In HNSCC, increased EGFR gene copy number as measured by FISH is common and is associated with worse progression-free survival and overall survival. Our laboratory has shown that patients with increased EGFR copy number determined by FISH had a worse median time to progression (TTP) and time to death compared to the FISH negative patients.⁴⁹ The prognostic value of EGFR FISH analysis was confirmed by another independent study.⁵⁰

3. Molecular characterization using genomic profiling

Comprehensive analyses of gene expression patterns of individual tumors can be achieved with DNA microarray technologies. Using this approach, many tumors have been classified into several relatively homogenous groups based on their gene expression patterns. Identification of subsets of HNSCC patients based on molecular characteristics of the tumors provided insight that may alter treatment based on their tumor biology. To search for subtypes of HNSCC tumors, we performed an “intrinsic” analysis to identify those genes whose expression was stable and which optimally reflected patterns of expression intrinsic to the tumors.⁵¹ Using this gene set, 60 tumors were analyzed using a two-way average-linkage hierarchical clustering analysis and the cluster analysis suggested the presence of at least four distinct groups. One of the groups was associated with a poor prognosis. However, routine use of array technology has been limited by the need of frozen tumors. This technical limitation is recently overcome by the development of assay that can utilize formalin-fixed paraffin-embedded tissues for the assay and it is expected to ease the validation of this assay using archived specimens (Figure 3).⁵²

4. Detection of Epstein Barr Virus as a prognostic biomarker

Nasopharyngeal carcinoma (NPC) is a specific carcinoma of the head and neck which is localized to the epithelial lining of the nasopharynx. With regard to epidemiology, treatment, and potential biomarkers, NPC is a distinct entity compared to other HNSCCs. Although relatively rare in the United States and Western Europe, NPC is endemic in certain areas of the world, including Southeast Asia and the Mediterranean. The role of EBV in the pathogenesis of this disease, particularly in endemic populations, makes this virus an attractive candidate as a clinically useful biomarker. The EBV genome is present in the tissue of nearly all non-squamous NPC (WHO Types II and III). Latent membrane protein (LMP-1) is considered to be the most important EBV gene product as far as transformation is concerned. Quantitative analyses of anti-EBV serologies and EBV DNA have been shown to be clinically useful for early detection, disease monitoring, and prognosis.

As Pap smears are used for cancer of the uterine cervix detection, several studies have examined the role of nasopharyngeal brush specimens for early NPC diagnosis. Using purely histological techniques, the sensitivity of cytology was 71% in one group of 98 cases which were suspicious for NPC.⁵³ The addition of PCR to cytology improved the sensitivity. Brushings from 21 patients with newly diagnosed NPC and 149 control subjects were sent for PCR for EBV. 19 of 21 patients with NPC were positive by PCR, while only 2 of the 149 control subjects were positive. This resulted in a sensitivity of 90% and a specificity of 99%.⁵⁴ Given the prevalence of EBV in humans, screening for NPC through PCR for EBV may be very low yield, with

many false positives. If EBV LMP-1 and the Epstein-Barr Nuclear Antigen gene (EBNA) are used as the targets for PCR, NPC can be diagnosed via nasopharyngeal swab with a sensitivity of 91.4% and a specificity of 98.3%. The utility of these two genes in NPC screening has yet to be evaluated.

EBV is a common infectious agent, and most humans carry IgG antibodies as a marker of past infection, complicating screening through EBV serologies. The presence of IgA antibodies against the EBV capsid antigen (VCA-p18) and neutralizing antibodies against EBV-specific DNase were found to be strong predictors for the development of NPC in one Taiwanese prospective cohort study.⁵⁵ After adjustment for age and family history, the relative risk of NPC in subjects with both markers, and one marker, was 32.8 ($p<0.001$) and 4.0 ($p=0.003$) respectively, when compared to subjects with neither marker. However, the yield is low: only 2 of 47 patients (4.5%) positive for both markers developed NPC over a 16-year period.

Using real-time quantitative PCR (RTQ-PCR), circulating cell-free EBV DNA is detectable in the plasma of patients with NPC, but not in controls. In fact, 95% of patients with NPC will have detectable EBV DNA at diagnosis.⁵⁶ The copy number by PCR increases with a corresponding increase in tumor burden, and decreases to undetectable levels with treatment related tumor regression.⁵⁶ Cell-free EBV DNA may prove to be a useful biomarker for NPC screening, measurement of response to treatment, and detection of recurrence.^{56,57} Plasma EBV DNA load is an independent prognostic factor, especially in predicting for metastatic potential.⁵⁸ When combined with the TNM staging of the tumor, the prognostic value of circulating EBV DNA improves. Early stage (I, II) NPC with high circulating EBV levels behaves similarly to advanced stage (III, IV) NPC with low circulating EBV, as far as overall survival is concerned.⁵⁹

Although promising, RTQ-PCR assessment of EBV DNA is not without technical difficulties. Different segments of the same viral DNA may have different sensitivities with this PCR assay and primers must be chosen accordingly. There has been a large amount of intra-group variability using the same primer/probe sets and conditions, bringing reproducibility and standardization of the techniques into question. Finally, DNA in stored blood will degrade, making retrospective analysis of samples difficult.⁶⁰

IV. Molecular tests for prediction of treatment response in squamous cell carcinoma

1. Expression of ERCC1 as a predictive marker of cisplatin response

Platinum-containing chemotherapeutic agents actuate their cytotoxic effects through covalent binding to the DNA molecule. Tumors which have increased DNA repair capabilities are more resistant to the effects of platinum agents. The excision-repair cross-complementation group 1 (ERCC1) is a rate-limiting enzyme in the nucleotide excision repair (NER) pathway. This DNA-repair protein is responsible for removing platinum containing DNA adducts. In a large study of NSCLC patients, high levels of ERCC by IHC were associated with resistance to platinum-based chemotherapy, as well as (paradoxically) an improved overall survival when compared to low ERCC1 (Figure 4).⁶¹ In two small studies of HNSCC patients, tumors with positive ERCC1 by IHC had worse survival and early progression after definitive cisplatin based chemoradiotherapy, compared to ERCC1 negative patients.^{62,63} Given that cisplatin is the standard of care in HNSCC, ERCC1 measurement may have predictive value in the treatment.

2. Hypoxia as a marker of radiation sensitivity

As tumors outgrow the blood supply, hypoxia and necrosis occur. In general, human solid tumors are poorly oxygenated compared to normal tissue, and it is estimated that over half of solid tumors have median oxygen levels of less than 10mm Hg. The adverse effect of cellular hypoxia in the treatment of cancer is well documented. Hypoxic tumors have an increased malignant phenotype, a higher propensity for metastases, and a much poorer prognosis. Tumors with very low pO₂ values (less than 7mm Hg) develop genomic changes such as point mutations, chromosomal aberrations, gene amplification, and polyploidy, leading to angiogenesis, invasion, and metastasis. This Darwinian selection leads to harder, resistant clones that can survive the harshest conditions.⁶⁴

This process of natural selection in the microenvironment has a direct bearing on treatment, as the tumor cells which can withstand hypoxia tend to become resistant to standard treatment. A threefold increase in radiation dose is required for hypoxic tumors compared to non-hypoxic cancers.^{65,66} Hypoxia leads to a cellular quiescence, rendering affected cells resistant to most standard chemotherapeutic agents.⁶⁷ Cytotoxic agents act best upon proliferating cells, rather than dormant hypoxic cells. It is also postulated that chemotherapy is poorly delivered to hypoxic regions of tumors with poor blood supply.⁶⁸ Given the inherent toxicities of intensified treatment modalities, it would be beneficial to know the degree of hypoxia, as this would affect the overall treatment plan. Traditionally, the “gold standard” for hypoxia determination is Eppendorf electrode oxygen measurement, which is impractical and invasive in the large majority of patients.⁶⁸ Using tissue microarray and proteomic techniques, the search for more practical endogenous markers of tissue hypoxia is being actively investigated. The role of markers of hypoxic response must be considered with a certain degree of caution. There is a body of evidence showing that tumor hypoxia and/or anoxia is heterogeneously distributed within the mass of a solid tumor, and there is variation from tumor to tumor.⁶⁴ A single biopsy or serum measurement may not accurately reflect the level of hypoxia in a large tumor.

2.1. Hypoxia Inducible Factor 1 α —The transcription factor hypoxia-inducible factor 1 α (HIF-1 α) is a major regulatory protein in the cellular response to hypoxia. HIF-1 α is induced by and rapidly accumulates under hypoxic conditions. After translocating to the nucleus, this protein binds to hypoxia-responsive elements (HREs) of hypoxia regulated genes. These genes include VEGF, erythropoietin, glucose transporter 1 (GLUT1), nitric oxide synthase (NOS), lactate dehydrogenase A (LDH-A), the lysyl oxidase family (LOX) and carbonic anhydrase IX, among others. In HNSCC patients treated with chemoradiotherapy, high HIF-1 α expression has been associated with a poor prognosis, with a higher rate of metastasis and a shorter DFS and OS.^{69–71} In these studies, HIF-1 α overexpression was determined using IHC.

Interestingly, improved survival was shown in a multivariate analysis of surgically treated patients with HIF-1 α overexpression.⁷² However, HIF-1 α levels have been shown to increase via oxygen-independent processes in cancer cells, and it is considered a non-specific marker for hypoxia. The gene products of HIF-1 α activation may serve as better surrogate biomarkers.

2.2. Carbonic Anhydrase IX—Carbonic Anhydrase IX (CA IX) is a transmembrane carbonic anhydrase which reversibly hydrates carbon dioxide to carbonic acid. CA IX is tightly regulated by the HIF-1 α pathway, and has been shown to be overexpressed in many tumor types. In both cell lines and archived tissue, CA IX was shown to have the most dramatic induction among the hypoxia-induced genes.⁷³ High expression by IHC of tumor tissue predicted poor survival in a variety of tumor types, including HNSCC.⁷⁰ Like HIF-1 α , the role of CA-IX as a prognostic marker is unclear, mainly due to the heterogeneous nature of hypoxia in HNSCC (as well as nasopharyngeal carcinomas).^{70,74,75} CA IX can also be induced by oxygen-independent factors.

2.3. Osteopontin—Osteopontin (OPN) is a tumor associated phosphoglycoprotein that has been studied as a plasma marker of tumor hypoxia since the mid-1990s. Measurement of serum OPN levels by ELISA in HNSCC patients correlates with high HIF-1 α expression, high VEGF expression, and low hemoglobin levels. A serum marker such as OPN may provide a clearer picture of global tumor hypoxia, avoiding sampling errors encountered in biopsy specimens. There is a large amount of variation in OPN levels measured using commercially available ELISA kits, and absolute levels of OPN must be interpreted with caution.⁷⁶

2.4. Lysyl Oxidase—The Lysyl oxidase (LOX) class of proteins is upregulated by HIF-1 α during the hypoxic response. This class of proteins plays a key role in regulation of the extracellular matrix, via stabilization of elastin and collagen fibers. Microarray studies have shown these proteins to be overexpressed in hypoxic human breast cancer and HNSCC lines, and it appears to have a prominent role in metastasis rather than primary tumor growth. HNSCC patients with high expression of LOX have been shown to have decreased survival and increased metastases when compared to patients with low levels of expression. Furthermore, tumor cells which produce LOX short hairpin RNA (thus inhibiting LOX activity) have a significant reduction in the development of metastases, although no effect on primary tumor growth was seen.⁷⁷

3. Response prediction of EGFR inhibitors

In many ways, the era of targeted cancer treatment began with Dr. Stanley Cohen's description of the epidermal growth factor (EGF) in the 1960s. Some thirty years later, the therapeutic benefit of the small molecule EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, was demonstrated in non small cell lung cancers (NSCLC). Later, EGFR was shown to have clinical importance in the treatment of colorectal cancer, through the use of the monoclonal antibody cetuximab. In recent years this anti-EGFR antibody cetuximab has come to the forefront in the management of HNSCC.

By definition, targeted agents would not benefit all patients without the pertinent genetic aberration. Therefore, there have been numerous studies to examine the predictive markers of response for EGFR inhibitors. In NSCLC, activating mutations in the EGFR kinase domain and increased EGFR gene copy numbers determined by FISH have been associated with a better response and survival with gefitinib treatment.^{78,79} However, these mutations occur rarely in HNSCC.^{80,81,49} EGFR staining by IHC has not been shown to correlate with the efficacy of EGFR targeted therapy in both HNSCC and colorectal cancer.^{82–84} In a recent study by Agulnik *et al.*, 37 samples from recurrent or metastatic HNSCC patients who were treated on phase I/II trial of erlotinib and cisplatin were examined for EGFR levels, its downstream proteins, and markers of angiogenesis and apoptosis. Among these markers, high EGFR copy number determined by FISH was correlated with a better response to erlotinib. When paired tumor biopsies from pre- and during-treatment were compared, decreased phospho-EGFR, phospho-NF- κ B and p27 levels during the treatment were associated with prolonged TTP and OS. When skin biopsies were compared, decreased phospho-EGFR was correlated with increased OS which suggests that skin biopsy may be used as a surrogate marker of response.⁸⁵ However, due to the small sample size, none of these markers met statistical significance and further studies are required for clinical application.

Although somatic mutations in the tyrosine kinase of EGFR domain occur rarely in HNSCC, a truncation of the extracellular, ligand-binding domain is thought to be a much more common event. This mutation results in an in-frame deletion of exons 2–7, and is termed EGFR variant III (EGFRvIII). It results in a weakly constitutively phosphorylated protein, which is ligand independent. EGFRvIII is expressed only in cancer cells, and always in the presence of wild-type EGFR. Recent data demonstrated the EGFRvIII mutant in 42% of HNSCC tumors by

immunostaining at RT-PCR. As this mutation results in ligand-independent constitutive activation of EGFR, EGFRvIII abrogates the response to EGFR specific monoclonal antibodies such as cetuximab.⁸⁶ EGFRvIII status may have predictive value in HNSCC, especially in light of the prominent role of cetuximab treatment in this disease, but this hypothesis remains to be tested.

4. Response prediction of anti-angiogenic agents

Angiogenesis is critical for primary tumor growth and for metastatic spread.⁸⁷ Vascular endothelial growth factor (VEGF) is a key regulator of new blood vessel formation in both normal endothelial tissue and in a wide variety of malignant tissues. VEGF exhibits its effect on the tumor microvasculature through high affinity binding with the tyrosine kinase receptors, vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and VEGFR-2). Increased expression of VEGF has been noted in many human cancers, including lung, renal cell, pancreatic, colorectal, and breast. VEGF is an endothelial-cell-specific mitogen which promotes tumor growth by inducing the proliferation and differentiation of vascular endothelial cells, increasing capillary permeability, and inhibiting endothelial cell apoptosis. Like EGFR, increased VEGF expression is associated with a worse prognosis, and VEGF serves as an independent prognostic factor in several tumor types. When combined with cytotoxic regimens, bevacizumab, a humanized monoclonal antibody against VEGF, has been shown to prolong overall survival in lung and colorectal cancers,^{88,89} and progression-free survival in breast and renal cell carcinomas.^{90,91} To date there are only limited data for the role of bevacizumab in HNSCC but appear to be promising.

The most commonly used method to measure the activity of anti-angiogenic agents is dynamic contrast-enhanced (DCE)-MRI; however, the role of imaging studies will be discussed elsewhere in this issue and the role of VEGF/VEGFR detection will be discussed here. The prognostic implications of VEGF in HNSCC are controversial. Several retrospective studies showed no correlation between VEGF levels and prognosis,⁹²⁻⁹⁵ while others have shown that high VEGF levels predict for shorter progression free survival, shorter overall survival, and a higher rate of disease recurrence.⁹⁶⁻⁹⁸ A recent meta-analysis of 12 studies involving 102 patients suggested a trend correlating VEGF positivity with worse overall survival.⁹⁹ Another study demonstrated that co-overexpression of VEGF and VEGFR-2 by IHC was associated with a higher tumor proliferation rate and worse survival. This data suggests that an autocrine VEGF loop may play a role in tumor survival, and VEGFR-2 mediates this loop.⁹⁹

Most early studies used IHC to measure VEGF expression, which is a semi-quantitative method. As in the detection of EGFR, IHC is not considered an adequate method for the detection. Using ELISA to quantify VEGF levels, one study showed that VEGF expression is associated with poorly differentiated head and neck cancers, higher rates of disease recurrence, metastatic disease, and shorter progression free survival.⁹⁸ Other studies have used laser capture microdissection followed by RT-PCR to compare VEGF mRNA levels in HNSCC with normal mucosa. A greater than seven-fold increase was found in the cancer samples.¹⁰⁰ Compared to IHC or ELISA, laser capture microdissection is a much more labor intensive process, diminishing its clinical utility.

V. Conclusion

When properly integrated to the current clinical parameters in the management of HNC, molecular diagnostic tests can offer the tremendous potential to stratify the risks of developing HNC, poor clinical outcome and to tailor treatment regimens as more treatment options are available in HNC. There have been palpable progress in the field recently but readily available diagnostic tests are few with several reasons. For reliable assay development, each assay must

utilize clinically available tissue materials. When the assay requires an extensive tissue preparation, it often fails when large samples are needed for validation in a multi-institutional retrospective study due to lack of sample availability. Also, each assay must be scrutinized for sensitivity, specificity and clinical applicability. If the assay does not add or are not an independent factor from the currently available clinical parameter, the impetus to developing a commercial assay is low. For the development of targeted agents, the clinical specimen collection and correlative studies must be initiated in the early development. Before commercial development, each assay must be rigorously evaluated and validated for clinical benefit.

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Figure 1.

Representative Case of Oropharyngeal Squamous-Cell Carcinoma That Was Positive for HPV-16 on In Situ Hybridization. Panel A shows fronds of in situ carcinoma, and Panel C shows nests of deeply invasive tonsillar carcinoma (both panels, hematoxylin and eosin). HPV-16 is visualized as hybridization signals (brown dots) within the tumor-cell nuclei in the corresponding right-hand images (Panels B and D, respectively) (Originally published by New England Journal of Medicine).¹⁸

Figure 2. Epidermal growth factor receptor (*EGFR*) gene copy number evaluation by fluorescent in situ hybridization. (A) gene amplification, (B) cluster gene amplification, (C) low polysomy, (D) trisomy, (E) normal disomy, and (F) high polysomy (*EGFR*, SpectrumOrange, Centromere 7 SpectrumGreen) (Originally published by American Society of Clinical Oncology).⁴⁹

Figure 3. Comparison of DNA microarray signatures generated from 29 formalin-fixed and 6 matched-frozen head and neck squamous cell carcinomas. The matched samples are denoted with blue bars (Originally published by American Association for Cancer Research).⁵²

Figure 4. An ERCC1-Positive Squamous-Cell Carcinoma (Panel A) and an ERCC1-Negative Squamous-Cell Carcinoma (Panel B) (Originally published by New England Journal of Medicine).⁶¹