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Advances and Perspectives in the Molecular Diagnosis of Head and Neck Cancer

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Abstract

Background—Head and neck squamous cell carcinoma (HNSCC) is a debilitating and lethal disease. Despite significant advances in radiotherapy and surgical management, the 5-year survival rate for head and neck cancer has remained a dismal 50%. Advances in early detection have been made, but to improve patient outcomes better biomarkers and targeted therapeutic agents are needed. Novel biomarkers can improve early detection and provide data to optimize therapeutic strategy and patient survival, and could lead to potentially effective targeted therapies.

Objective—Report the advances in the discovery of novel biomarkers for HNSCC, and review the potential utility of biomarkers in the molecular diagnosis of HNSCC.

Methods—A review of the English literature (PubMed) from 1980 to 2009.

Results/conclusion—Currently the most widely accepted biomarker for HNSCC is high risk HPV status. EGFR is another promising biomarker, however, further research is necessary to determine its prognostic benefit. A large number of promising biomarker candidates are currently being evaluated including epigenetic, expression, and genomic based markers. Studies to validate the sensitivity and specificity of these biomarkers in clinical samples from adequately powered prospective cohorts are needed for successful translation of these findings into potential molecular diagnostic, prognostic, and therapeutic biomarkers for HNSCC.

Keywords

head and neck cancer; molecular biology; diagnosis; biomarkers; human papillomavirus; epidermal growth factor receptor; loss of heterozygosity; epigenetics; DNA methylation; proteomics; RNA; microRNA

1. INTRODUCTION

Over 48,000 new cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed in the United States annually, with a mortality rate close to 12,000 deaths annually. This

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corresponds to >4% of all new cancer cases and 2% of all cancer deaths in the United States each year. Overall, this disease affects more than 500,000 people in the world [1,2]. Despite aggressive multidisciplinary treatment approaches along with significant advances in surgery, chemotherapy and radiotherapy, the survival rate has only moderately improved, with the 5-year overall survival remaining at 50% over the past 30 years. Most patients with premalignant lesions and early stage cancers have a high cure rate and survival, but the vast majority of stage III and IV cases are fatal partly due to the relatively high local and regional recurrence rates. Unfortunately, only 30% of HNSCC in the United States are diagnosed at an early clinical stage [1-3]. Early detection of HNSCC could improve clinical outcomes, but there is no definitive evidence that widespread population screening using conventional methods like head and neck examination, fiberoptic endoscopy, and/or staining with direct visualization decreases mortality from HNSCC [4].

To improve patient outcomes, the development of reliable biomarkers and more effective therapeutic agents are necessary. The use of biological markers in body fluids for molecular detection of cancer has been the subject of an increasing number of translational studies with the intent to improve overall screening accuracy and cost-effectiveness. Body fluids can potentially carry whole cells as well as protein, DNA, and RNA species that allow for detection of cellular alteration in cancer. Previously published examples of body fluids used for detection include sputum analysis for lung cancer, urine for urologic tumors, saliva for HNSCC, breast fluid for breast cancer, and several reports using serum and/or plasma for other malignancies [5-16].

The goal of any robust molecular detection and diagnostic strategy is to identify pre-malignant and malignant tumors early, but also to be able to use available biomarkers to prognosticate and risk stratify patients, as well as predict therapeutic response to conventional treatments and treatment failures. Many examples can be found in other tumor models, most notably O^6-*methylguanine-DNA methyltransferase* (*MGMT*) promoter methylation status in glioblastoma and the presence of *estrogen* (*ER*), *progesterone* (*PR*) and *human epidermal growth factor receptor 2* (*HER-2*) receptors in breast cancer. *MGMT* promoter methylation status is a key feature of glioblastomas that characterizes the response of tumors to temozolamide and radiotherapy treatment in approximately a third of tumors. Methylation of this molecular biomarker is associated with not only marked improvement in treatment response, but also increased survival [17]. Similarly, *ER*, *PR* and *HER-2* receptor positivity in breast cancer dictates disease behavior, prognosis and treatment selection [18-20]. Tumors expressing *ER* and *PR* receptors respond frequently to endocrine therapy with Tamoxifen and carry a much better prognosis. Most of these patients can avoid aggressive and toxic chemotherapy agents while still being effectively treated for the disease. *HER-2* status is a marker of more aggressive disease with higher recurrence rate and frequent resistance to standard chemotherapeutic therapies, but at the same time can stratify patients to tailored therapy with Herceptin, a *HER-2* specific antibody [21,22].

Molecular and cellular biology are promising fields of study in HNSCC that continually lead to the discovery of novel biomarkers and potential therapeutic targets. In addition, advancements in head and neck cancer epidemiology, genetics, epigenetics, proteomics, RNA and microRNA, along with the rapid development of high throughput microarray technology and powerful bioinformatics that allow integration of complex data and molecular pathways, all help elucidate the complex picture of HNSCC tumorigenesis. The use of molecular biomarkers, multiple gene detection panels and targeted therapeutics are becoming a reality in everyday clinical practice. However, translational research studies need to continue as further insight into the molecular basis of head and neck cancer will yield advances in early screening and diagnosis, and ultimately hopefully translate into improved clinical outcomes. In the

following sections, we will review and discuss recent discoveries in HNSCC tumor biology, and their impact on potential molecular screening and diagnosis of head and neck cancer.

2. MOLECULAR PROGNOSTIC FACTORS

2.1 Human papillomavirus infection in head and neck cancer

Tobacco and alcohol consumption are two primary environmental risk factors associated with the development of HNSCC. In the last two decades new insight into head and neck cancer epidemiology recognized that infection with viral pathogens such as human papillomavirus (HPV) play an important causal role in the pathogenesis of a unique subset of oropharyngeal HNSCC, similar to the role HPV infection plays in cervical cancer [23-29]. Human papillomavirus is present in up to 60% of patients with oropharyngeal HNSCC and confers a favorable prognosis in terms of recurrence and mortality, and is a distinct established entity that can be reliably diagnosed.

Briefly, human papillomavirus is a ~7.9 kb, non-enveloped, double-stranded, circular DNA virus that has a specific tropism for squamous epithelium. There are 13 known high-risk HPV types that can transform cells that may lead to cancer, but only high-risk HPV subtypes 6, 16, 18, 31, 33 and 35 have been identified as playing a role in the development of oropharyngeal HNSCC [24,27,30-38]. Regardless of the study population, high-risk HPV16 accounts for the overwhelming majority (90–95%) of HPV-positive tumors [38]. The *E6* and *E7* oncoproteins contained within the viral genome are able to disrupt the function of *Rb* and *p53*, which are well known tumor suppressor genes, leading to development of a malignant phenotype [39].

Most of the HPV related tumors are primarily found within the lingual and palatine tonsils, otherwise known as the Waldeyer's tonsillar ring, and they distinguish themselves as a separate entity from other HPV negative HNSCC tumors [33]. HPV positive HNSCC patients are usually non-smokers and non-drinkers and present at a more advanced stage at initial diagnosis [24,31,40]. In a study restricted to patients with oropharyngeal cancers, nonsmokers were approximately 15-fold more likely to have a diagnosis of HPV positive HNSCC than smokers [41]. More recently, several clinical studies showed that HPV is an independent risk factor, and does not merely modify the riskof HNSCC associated with tobacco and alcohol exposure [42]. Patients with HPV positive HNSCC are approximately 5 years younger than HPV negative HNSCC patients with equal distribution among the sexes [30-32]. Risk factors for HPV related HNSCC include a high lifetime number of vaginal-sex partners of 26 or more as well as a high lifetime number of oral-sex partners of 6 or more, and seropositivity for HPV16 viral capsid protein antibodies carries a 15-fold increased risk for HNSCC [42].

HPV status of tumors also improves our ability to provide an accurate prognosis. HPV positive patients have a much higher response to therapy than non-HPV related HNSCC, which translates into much improved clinical prognosis and survival. In the majority of studies, patients with HPV positive tumors have as much as a 60–80% reduction in risk of dying from their cancer when compared with the HPV negative patient after controlling for other risk factors [24,41,43-46]. HPV positive patients also had a much higher response rate to radiation, chemotherapy and chemoradiation treatments, and a significantly higher 2-year overall survival (95% vs 62%) [40,41].

Through detailed epidemiological studies, the establishment of the causal relationship between HPV infection and oropharyngeal cancer has improved the ability to diagnose and locate disease even in occult primary tumors. Molecular detection of HPV in metastatic cervical lymph nodes is a highly effective strategy for localizing the site of tumor origin to the oropharynx [47]. Quantitative polymerase chain reaction (PCR) and other molecular techniques were also used to evaluate the presence of HPV DNA in exfoliated oral mucosal

cells from patient surveillance salivary rinses. HPV-16 positivity in surveillance salivary rinses had a sensitivity and specificity for recurrence diagnosis of 50% and 100%, respectively. HPV 16 DNA could be detectedin follow-up surveillance salivary rinses on average 3.5 months before the definitive clinical diagnosisof disease recurrence. HPV 16 positive surveillance salivary rinses were a marker for poor prognosis due to locoregional recurrence and distant metastasis, and patients were at high risk for recurrence [48]. HPV positivity is therefore a robust molecular biomarker for a subset of head and neck cancers that has potential to be a used as a valuable surveillance molecular biomarker.

Oropharyngeal HNSCC is therefore a distinct subsite entity that can be reliably diagnosed using molecular techniques looking at HPV status; most robust molecular diagnostic and prognostic marker to date. In terms of screening, there is some controversy as there is no unanimously accepted specific antigenused for HPV detection, making comparison of clinical data and uniform clinical trial design difficult. The most widely applied detection methods are PCR based amplification of the HPV genome. However, these methods are extremely sensitive as they can detect even a few copies of DNA per sample, resulting in false-positive results. Moreover, HPV-DNA presence does not prove viral causation for the neoplastic transformation and may reflect only a transient infection, as most studies show only a fraction of HPV DNA positive tumors expressing viral proteins [49]. Hence HPV genome integration, transcription and detection of viral proteins to identify biologically active virus in tumors is key. Some studies use antibodies specific for the *L1* capsid proteins, while other propose the use of *E6* and *E7* oncoprotein specific antibodies to detect HPV [50]. The biological relevance of this is yet to be resolved, though detecting biologically active virus in tumors is the key for theragnostic clinical studies. Commercially available in situ hybridization assays for HPV DNA are for now the gold standard tests for clinical classification of an HPV positive tumor as described by Gillison [51].

Currently HPV is the most valid molecular diagnostic test for HNSCC and given the favorable response to therapy and improved prognosis, the current American Joint Committee on Cancer staging system for head and neck cancer may be modified to reflect these important differences. HPV testing is becoming a part of a molecular staging system for HNSCC. Possible future diagnostic tests that would likely have high specificity but low sensitivity for a diagnosis of HPV-associated HNSCC will include the detection of HPV16 DNA in plasma and saliva [48,52]. Other screening tests like fluorescence in situ hybridization (FISH) on papanicolaou smears obtained directly from tumors and HPV16 *E6* and *E7* seroreactivity are other tests currently being investigated.

In similar fashion, *Bcl-2* may be another biomarker that proves to be an important HNSCC prognostic marker. *Bcl-2* acts downstream from the HPV dysregulated *Rb* and *p53* pro-apoptotic pathway, by blocking *p73*, a tumor suppressor gene related to *p53* that shares many of its proapoptotic functions [53]. Recently, a study by Rocco *et al*. showed that tumors overexpressing *Bcl-2* have a 6-fold greater risk of treatment failure with cisplatin-based chemoradiation treatment. Immunohistochemical assessment of *Bcl-2* in pretreatment biopsies predicted response of oropharyngeal HNSCC to therapy, and could prove to be another independent prognostic marker besides HPV as there was no correlation between HPV infection and *Bcl-2* status [54]. More research is required to determine the potential interactions between HPV and *Bcl-2* status in predicting outcome in oropharyngeal HNSCC.

2.2 Epidermal growth factor receptor

The *epidermal growth factor receptor* (*EGFR*) is a widely studied oncogene in HNSCC. This receptor tyrosine kinase belongs to the *ErbB* family of cell surface receptors and has many downstream signaling targets associated with tumorigenesis. Once activated, the receptor can signal via multiple pathways such as *MAPK*, *Akt*, *ERK*, and *Jak/STAT*. These pathways are

related to cellular proliferation, apoptosis, invasion, angiogenesis, and metastasis [55-57]. In general, expression of *EGFR* is a normal finding in many tissues including the dermis, gastrointestinal tract and kidneys. However, dysfunction in the signaling of this receptor and its downstream targets commonly occurs in most epithelial cancers, but also in over 80% of HNSCC cases [56-58]. *EGFR* is a promising marker and prognosticator of disease, and the understanding of its molecular biology has led directly to biologically significant targeted therapies.

Initial studies found that *EGFR* was upregulated in HNSCC cell lines and in a high percentage of primary HNSCC tumors [59-61]. Furthermore, histopathologically normal mucosa adjacent to cancer had a high degree of overexpression, and upregulation of *EGFR* is an important step in the transition from dysplasia to HNSCC [62,63]. It seems that *EGFR* is an important step in tumorigenesis and a useful prognostic molecular marker since elevated levels of expression confers poor survival [64]. In 2005, the continuous hyperfractionated accelerated radiotherapy (CHART) head and neck cancer phase III clinical trial, demonstrated that overexpression of *EGFR* in pre-treatment biopsies is a robust biomarker for improved response to radiotherapy and could serve as a predictive marker for therapeutic response, encouraging further development of *EGFR* targeting combined with radiotherapy [65].

Follow-up studies showed that *EGFR* is also a potential therapeutic target for tyrosine kinase inhibitors as well as other anti-*EGFR* targeted molecules [65-67]. Cetuximab is one of the most well studied monoclonal antibodies directed against *EGFR.* A recently published Phase III clinical trial examined the effects of this drug in conjunction with radiotherapy in the treatment of locoregionally advanced HNSCC. This study demonstrated an overall survival benefit (49 vs. 29 months) and increased duration of locoregional control (24.4 vs. 14.9 months) in the cetuximab plus radiotherapy arm versus the arm receiving radiotherapy alone. This was the first randomized study showing a survival benefit with an *EGFR* targeting agent in locally advanced HNSCC [66,67].

EGFR continues to be an important area of ongoing research, especially focusing on tumor specific response to therapy and survival outcome, but also detection. The challenge for molecular diagnostics is that there is no uniform assay or definition for aberrant expression of EGFR making it difficult to standardize results and design future diagnostic studies.

2.3 Genetics and loss of heterozygosity

The previously formulated genetic progression model for HNSCC states that there is a relatively common pattern of DNA allelic loss during the progression from premalignant to malignant phenotype [68]. Using simple PCR-based molecular techniques, one can identify these losses of genetic material, represented by complete deletion, or loss, of one allele, otherwise known as loss of heterozygosity (LOH). Tumor suppressor genes (TSG) may be in the area of loss and thus would make the cell more susceptible to dysfunction of these genes, which could lead to cancer development, especially if the same gene already contains a deleterious mutation on the matching allele.

One of the most promising areas currently under investigation is the ability to analyze premalignant and tumor margin tissue for regions of LOH known to be associated with increased risk of progression to carcinoma. Several regions of chromosomal loss are commonly found in HNSCC. One of the earliest and most common of all genetic changes associated with HNSCC tumorigenesis is the loss of chromosome region 9p21–22 which occurs at a frequency of 70% [69]. Loss of chromosome region 3p also occurs but less frequently. Thirty percent of the earliest precancerous lesions also exhibited loss at either 9p21 or 3p [70]. Such studies indicate that LOH is a common event in both malignant and premalignant lesions that could carry significance in early diagnostics and tumor surveillance.

Patients with premalignant mucosal lesions demonstrating loss of chromosomes 9p21 and 3p14 more frequently progressed to HNSCC compared to patients without LOH at these loci [71]. Benign premalignant lesions that harbor these genetic loses had a 3.8-fold increased risk of progression to cancer. When additional chromosomal losses are acquired including 4q, 8p, 11q, or 17p, the risk increased 33-fold [72]. Another study looking at cumulative genetic loss and its relationship with progression to cancer screened premalignant lesions for LOH for several markers including 3p21, 8p21-23, 9p21, 13ql4.2, 17p13.1, and 18q21.1 and showed that having LOH in two more of these regions carried a 73% probability of developing cancer within 5 years [73]. More recently, several smaller studies looking at tumor margins that are histologically normal have also shown that LOH of the field of cancerization frequently found in oral HNSCC may lead to cancer and that these transformed cells originated from the same clonal lineage [74,75].

Unfortunately, LOH screening panels are currently not available commercially. Large scale LOH testing, however, is in the developmental phase and being used in several screening and surveillance federally funded clinical trials for squamous cell carcinoma of the upper aerodigestive tract. In the future, it is clear that LOH testing will improve our ability to accurately diagnose and treat pre-microscopic disease, which is an important step towards decreasing the risk of recurrence and increasing overall survival in HNSCC patients.

3. MOLECULAR DETECTION STRATEGIES

3.1 Epigenetics

The field of epigenetics has greatly impacted our understanding of cancer biology. Epigenetics is defined as the stable inheritance of genetic information based on gene expression levels without changes in the genetic code. The heritable modifications of DNA occur through several pathways including alterations in DNA methylation and histone modification. These epigenetic alterations have been associated with cancer-specific gene expression differences in human malignancies, and are known to occur early in tumorigenesis [76].

Methylation of the 5′ carbon of the cytosine ring within cytosine–guanine dinucleotides (CpGs) by the enzyme class DNA methyltransferases is a commonly found epigenetic modification frequently studied in humans. CpG methylation occurs in close proximity to the transcriptional start site, leading to block transcription and recruit histone modifiers. This ultimately results in tightly packed heterochromatin and gene silencing that is both species and tissue specific [77]. As a novel mechanism of gene regulation, epigenetic control of tumor suppressor genes (TSGs) was quickly proposed as a potentially important mechanism of carcinogenesis [78-80]. Hypermethylation of CpG gene promoter regions has been primarily considered as the mechanism of TSG inactivation, but more recently several studies re-emphasize the importance of early studies of hypomethylation in tumor development and its potential for unmasking expression of putative oncogenes [78,81-89].

Sodium bisulfite treatment of DNA, which converts non-methylated cytosines to uracil, and more recent development of new assays such as quantitative methylation-specific (QMSP) and quantitative unmethylation-specific PCR (QUMSP) have further advanced our ability to evaluate the methylation status of tissue samples [87,90-92]. With these advances, many different TSGs in various tumor types have been shown to be down-regulated by hypermethylation, and the utilization of comprehensive whole genome profiling approaches to promoter hypermethylation has identified novel putative TSGs silenced by promoter hypermethylation [93].

Dysregulation of DNA methylation and the associated gene expression changes in tumors and pre-malignant tissues makes DNA methylation profiling an attractive target for molecular

studies, and since it is heritable but reversible, it also has great potential for identifying novel therapeutic targets [81]. The potential for quick real-time and high throughput analysis of samples makes these new findings ideal for study as novel molecular biomarkers not only in primary tissues but other biologic fluids and compartments. Rigorous testing is necessary on separate validation cohorts to calculate the sensitivity and specificity of these potential biomarkers.

Studies of promoter methylation in primary tissues have uncovered many putative TSGs in HNSCC including *p16*, *lhx-6* (*DIME-6*), ATM, p15, TIMP-3, MGMT, RARB-2, DAP-K, E-cadherin, Cyclin A1, RASSF1A, CDKN2A, CDH1 and *DCC*. These genes are known to function in pathways that control cell cycle progression, apoptosis, cell to cell adhesion, DNA repair and tumor invasion [94-108]. With the continued advent of new molecular techniques and whole genome screening strategies using array-based DNA methylation profiling, the list of TSGs that are silenced through promoter hypermethylation continues to grow at a rapid pace. New technology that utilizes such an array-based platform for genome wide epigenetic profiling has already been put to use to study distinct patterns of DNA methylation in HNSCC. This holds promise for integration of epigenetic and expression array data and continued discovery and validation of gene targets [109]. Successful screening and surveillance strategies involve the collection of genomic material from patients using minimally invasive approaches. This makes both oral rinses and serum analysis attractive options for patients with HSNCC. It is widely accepted that oral rinses harbor either naked DNA or shed cells that might harbor cancer DNA that could be used for detection or surveillance of HNSCC. The use of serum and salivary rinse DNA analysis has already been shown in limited cohorts with a small number of genes to successfully identify differential promoter hypermethylation patterns and can potentially predict the likelihood of developing metastatic disease [11,12,110-113]. Therefore, both salivary rinses and serum provide good mediums for collecting genomic content as a means of diagnosis and surveillance.

On a more global scale, unique hypermethylation profile panels give us the molecular ability to differentiate cancer from normal, but also to define certain specific cancer types [114,115]. Using these principles and the screening strategies mentioned above, an extended panel of promoter hypermethylation markers has demonstrated an improved ability to detect epigenetic changes associated with HNSCC in salivary rinses and serum. Using different combinations of these genes allows for improved detection of HNSCC by QMSP in both salivary rinses and serum compared with single markers, which holds promise for development of screening and diagnostics panels in HNSCC [116].

Evidence also supports a role for hypomethylation in tumor development, and global genomic hypomethylation has been reported in almost all solid tumors, including HNSCC [81-83,88]. A meta-analysis based on several different types of solid human tumors, showed an overall correlation between global hypomethylation and advanced tumor stage [83]. To date, only sporadic examples of promoter hypomethylation associated with unmasked expression of putative oncogenes have been reported, including *R-Ras* and *MAGE-A1* and *A3* in gastric cancer [85,86], the *Hox11* proto-oncogene in leukemia [89], *BCL-2* gene hypomethylation and high-level expression in B-cell chronic lymphocytic lymphomas [84], and rare activation of two *RAS* family members in colon cancer and small cell lung cancer [78]. These observations demonstrate that proto-oncogenes with tissue-specific expression may be inappropriately re-expressed in cancers via epigenetic alteration, including demethylation. A recent report uncovered aberrant activation of candidate proto-oncogenes via promoter demethylation in HNSCC as well as lung cancer [87]. Upregulation of these genes was shown to carry biological significance in tumor development and are yet another group of potential novel biomarkers that could hold promise for molecular detection and aid in the diagnosis of HNSCC.

3.2 Proteomics

Proteomics has emerged as one of the new avenues of research in cancer biology, and the impact in HNSCC remains to be seen. Proteomics involves the high-throughput global analysis of proteins within biologic samples. These assays have been applied in studying HNSCC samples and have shown promising results. A number of tumor-associated proteins were frequently found to be significantly altered in their expression levels in HNSCC tissues, compared with their paired normal mucosa, including *stratifin*, *stathmin*, *heat shock protein 27*, and *superoxide dismutase 2* (*SOD2*) [117-120]. The patterns detected and the particular proteins identified, from tissue and serum, can be used to characterize the tumors, as well as provide insights into the mechanisms involved in carcinogenesis [14,15,121]. In one study, supervised prediction analysis revealed excellent classification of healthy mucosa and tumor samples, with 94.5% and 92.9% samples correctly classified, respectively. Such proteomic profiling in conjunction with protein identification greatly outperformed histopathological diagnosis, and a significant association between aberrant protein profiles and tumor recurrence was found [119]. Elevated *SOD2* levels were also recently associated with lymph node metastasis in HNSCC and may provide predictive values for diagnosis of metastasis [122]. Serum analysis by protein profiles also proves to be robust in molecular detection of HNSCC with peak sensitivity and specificity of 83% and 100%, respectively [14,15,121].

There are some drawbacks and limitations to using proteomics to analyze valuable and limited clinical specimens. Often there are only subtle changes present in cancer that could be key in cancer signaling processes. Unfortunately, current protein detection is insensitive in detecting these subtle changes in oncoprotein expression and activation, and the process itself requires large numbers of cells from tumor specimens for analysis. The ability to detect specific proteins and their activation is likely to be highly useful in the development of new targeted therapeutics, as well as in monitoring their effectiveness and results. The field of proteomics continues to evolve and new technology is now available which has the potential to enable researchers to complete highly specific analysis of proteins from these limited or low-yield clinical samples. Fan *et al*. recently reported on development of a nanofluidic proteomic immunoassay to quantify total and low-abundance protein isoforms in nanoliter volumes, which promises to revolutionize the field of proteomics and potentially bring it one step closer for clinical diagnostic use [123].

There is much more that needs to be done in terms of the bioinformatic analysis of these assays and in the proper identification of the exact protein signatures. Once there is a better understanding of the data produced by proteomic studies, and the data is correlated with DNA and RNA expression profiles and other known genetic alterations, there is potential for development of a broader array of serum analyses whereby the diagnosis, response to therapy, and recurrence might be detected by a simple blood test. Active research investigation needs to continue in this area as the implications of specific results could have profound clinical and diagnostic implications.

3.3 RNA and MicroRNA

Similar to DNA and protein-based detection strategies, RNA, including microRNA (miRNA), may also be used for identification of altered gene expression patterns in cancer. Cancer-related nucleic acids can be isolated and detected in blood, urine, cerebrospinal fluid and saliva utilizing reverse transcription-PCR detection strategies, and have been used as biomarkers for cancer diagnosis [124-127]. As is true in high throughput analysis of DNA and protein samples, there is growing availability of powerful and cost-efficient microarray technologies that enable mass screening of messenger RNA (mRNA) and miRNA profiles.

Microarray analysis uncovered a large panel of human mRNA signatures that exist in saliva and suggested that salivary transcriptome analysis could be useful in diagnostics and surveillance of oral head and neck cancer patients [125]. Further work demonstrated that differentially expressed mRNA transcripts between cancer and normal patient salivary samples could be identified and used as potential biomarkers for cancer detection. Analysis showed that close to 1700 genes exhibited significantly different expression level in saliva between cancer patients and controls. Several salivary mRNA biomarkers for oral HNSCC were identified including *DUSP1, H3F3A, OAZ1, S100P, SAT, IL8* and *IL1B*. Aberrantly expressed mRNA transcripts exhibited at least a 3.5 fold elevation in cancer patients, and the combination of the biomarker panel yielded a sensitivity and specificity of 91% in distinguishing oral HNSCC from the controls [128].

With the discovery of miRNA molecules in *Caenorhabditis elegans* in 1993, a novel method of gene expression regulation was revealed. MicroRNA molecules are small ~22 nucleotide, non-coding RNA molecules that have been shown to regulate post-transcriptional gene expression by relatively nonspecific binding to the 3′-untranslated region of mRNA [129, 130]. These miRNAs are thought to be involved in a host of cellular processes including differentiation, apoptosis, and proliferation. MicroRNA have recently been an area of interest for a variety of human diseases including cancer. The expression profiles have been established for many different cancers and seem to be unique to each cancer [131]. Interestingly, it has been demonstrated that miRNAs with putative tumor suppressor function may also undergo epigenetic silencing in cancer [132,133].

MiRNAs play a large regulatory role and are a powerful tool in studying perturbations in gene expression. MiRNA arrays are now also being performed on HNSCC, but the interpretation of the results is still in its preliminary stages [134]. Nonetheless, there is increasing evidence of aberrant expression of miRNAs in HNSCC. When evaluating the expression pattern of 156 mature miRNAs in HNSCC of the oral cavity, *miR-133a* and *miR-133b* were significantly reduced in tumor specimens when compared with paired normal epithelial samples, resulting in activation of a potential oncogene *pyruvate kinase type M2* [135]. A more recent study indentified several miRNA alterations in primary HNSCC tissue samples that correlated with cell line studies showing biological significance [136]. These miRNA perturbations are being applied in the development of clinical biomarkers for HNSCC disease [137]. Avissar *et al*. used miRNA microarrays and separate validation to reliably confirm 4 differentially expressed miRNAs in HNSCC tissues. Using miRNA expression ratios, they found that the *miRNA-221/ miRNA-375* seems to be predictive of HNSCC with a sensitivity and specificity of 92% and 93%, respectively. It is important to note that miRNA profiles of cell lines can differ greatly from primary tissue as cell lines potentially develop variant miRNA signatures during the culturing process [138]. Hence it is critical that miRNA biomarker discoveries are performed in primary human tissue.

The interactions of the miRNAs can be difficult to predict, and each one may have several hundred to more than a thousand putative targets due to their relatively nonspecific binding to target mRNA. However, there is no question that they play a large role in the regulation of gene expression, and could potentially be utilized as molecular biomarkers in tissue as well as body fluids such as serum and saliva. This is an active area of investigation in many different cancer models, including head and neck cancer. Further study is necessary to look at miRNA use in diagnostic tests, prognostic significance or even as possible therapeutic targets.

4. CONCLUSION

Despite significant advances in clinical practice and treatment, the clinical outcomes in head and neck cancer only moderately improved. Early detection and novel robust screening

strategies are needed to improve survival and morbidity associated with this disease. Continued support for basic science and translational research is necessary for the identification and characterization of novel biomarkers in head and neck cancer as they may provide sensitive targets for molecular screening tests that ultimately will power molecular diagnostics and surveillance. The field of molecular biology has been growing exponentially, especially when it comes to understanding the increasing complexity of head and neck cancer. Several robust molecular markers of the disease have been found and are being actively validated; most notably HPV infection and *EGFR* status. These are the leading candidates for developing novel diagnostic and therapeutic strategies for HNSCC specifically. Further advancements in the molecular biologic understanding of HNSCC have significant potential for screening, diagnosis, prognosis, surveillance and treatment selection of patients with this disease. The ultimate goal of understanding the molecular biology of HNSCC is to try and apply this information to everyday clinical practice, similarly to the current utility of HPV and *EGFR*, in an effort to improve patient outcomes with the use of molecular markers for robust screening, prognostic assessment, treatment response and diagnostics, collectively referred to as theragnostics.

5. EXPERT OPINION

Steady development of new technology and novel techniques aimed at elucidating additional aberrant molecular alterations characteristic of HNSCC, including the advent of high throughput assays and the development of more sophisticated bioinformatics tools, help elucidate the complex tumorigenesis of HNSCC. With novel genome-wide molecular assays, the ability to detect these abnormalities has improved, and led to the discovery of many molecular biomarkers in HNSCC that can be used for molecular detection and diagnosis.

The goal of any robust molecular detection and diagnostic strategy is to identify pre-malignant and malignant tumors early, but also to be able to use available biomarkers to prognosticate and risk stratify patients, as well as predict therapeutic response to conventional treatments and treatment failures. Most of the molecular diagnostic strategies and risk stratification in HNSCC are concentrated on HPV and *EGFR* status of tumor tissues, while LOH and other genetic aberrations are proving important in the analysis and prognosis of pre-malignant lesions as is being studied in several ongoing clinical trials. HPV positivity in HNSCC confers a favorable prognosis in terms of radiation sensitivity, recurrence and overall mortality. Notably, HPV is a reliable biomarker that can be used to help diagnose HNSCC, but more importantly it can be used to risk stratify patients and help direct treatment plans based on the disease behavior and prognosis [23-29,40]. Currently, this is the most valid and robust molecular diagnostic and prognostic biomarker to date for HNSCC. Similarly, *EGFR* is also a promising marker and prognosticator of disease, and the understanding of its molecular biology has led directly to biologically significant targeted therapies with tyrosine kinase inhibitors and Cetuximab, though problems associated with these molecular therapies and their mechanism of action need to continue to be studied. Upregulation of *EGFR* is an important step in the transition from dysplasia to HNSCC [62,63], and its overexpression in pre-treatment biopsies can be a robust biomarker for improved response to various therapeutic modalities [64-67]. LOH is a common event in both premalignant and malignant lesions that could carry significance in early diagnostics and tumor surveillance. Patients with benign premalignant lesions that harbored HNSCC specific genetic loses and LOH had a significantly increased risk of developing cancer [72,73]. All of these are proving to be powerful molecular biomarkers based on tissue analysis, with great predictive power in terms of tumor behavior and treatment outcomes and we will see them used increasingly in clinical practice worldwide.

Similarly to tissue analysis, the use of molecular markers in body fluids has been explored in many tumor models, including HNSCC, with the intent to improve screening accuracy and

cost-effectiveness of diagnostic testing. Body fluids are therefore invaluable resources as they act as a surrogate test vessel that harbors biological markers that originate from primary tissue sources. Samples are easily prepared for high-throughput testing, while collection is minimally invasive for patients and requires only limited resources and staff training. Epigenetic DNA alterations and the real-time PCR methodology utilized for analysis allows for an objective, robust, and rapid assessment of these changes. This technology holds great promise with regards to molecular diagnosis using body fluid samples. Sputum from lung cancer patients can detect aberrant promoter methylation in patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis [10], and prospectively, a panel of hypermethylated genes is able to identify high-risk patients [139]. Similarly in HNSCC, the ability to group previously identified hypermethylated gene targets into detection panels has greatly improved the ability to detect and screen for epigenetic changes in both serum and salivary rinses [116]. Specificity reached as high as 97.1% but was unfortunately usually associated with low sensitivity, limiting its use in population-based screening, although several identified panels with high sensitivity but low specificity show promise for surveillance in a high-risk population.

Analysis of protein, RNA, and miRNA aberrations in tumors has also contributed to a growing list of potential HNSCC biomarkers, which have been used in multiple molecular detection panels performed on sample microchip technology. This also holds great promise for high-throughput real-time sample analysis for development of molecular diagnostic tests in body fluids; pending validation and testing. Proteomic profiling in conjunction with protein identification are already being verified in various biological fluids from HNSCC patients and are proving superior to histopathologic evaluation [119], and are reaching sensitivity and specificity levels up to 83% and 100%, respectively [14,15,121].

The increasing availability of microarray technology that enables mass screening of mRNA has uncovered a salivary transcriptome that is potentially both sensitive and specific for oral HNSCC, reaching a sensitivity and specificity of 91% [128]. Although these findings are based on a small sample size they continue to hold promise for the role of mRNA profiling in diagnostics as is true for miRNA. With increasing evidence of aberrant expression of miRNA in HNSCC, results from preliminary panel based arrays on tissue show sensitivity and specificity of up to 92% and 93%, respectively [138]. Active research investigation needs to continue in the molecular detection of body fluid as the implications of specific results could have great clinical implications in the realm of diagnostics, especially because of the minimally invasive nature of body fluid collection and its availability.

Many of these advances have lead to an increasing number of translational studies in the diagnosis, prognosis, and treatment of head and neck cancer. The end result is that molecular biomarkers, gene detection panels and targeted therapeutics are becoming a reality for the care of patients with HNSCC. This cannot be over emphasized as the statistics show only modest improvement in survival of patients with HNSCC over the last 30 years, despite aggressive multidisciplinary team treatment, including preoperative or postoperative chemotherapy and/or radiotherapy combined with surgical resection and reconstruction options. To further improve patient outcomes, more robust molecular biomarkers and the therapeutic agents that might result from them are necessary, especially if they improve early detection and diagnosis of lesions.

Detection of molecular biomarkers could be one of the integrative genomic tools that will help us to make this a reality in HNSCC. Currently, HPV and *EGFR* are the leading candidates for developing valid clinical diagnostic strategies for HNSCC. Several other screening panels composed of a variety of biomarkers and applied not only to primary tissue but also other biological fluids and genetic reservoirs such as salivary rinses as well as serum and plasma, have successfully shown great potential for molecular diagnostic use in HNSCC detection. The

clinical investigation of these applications is still just in its infancy, and with great promises comes the responsibility to carefully and rigorously test the validity of these findings. With continued advances in technologies that are both improving sensitivity and specificity while at the same time drastically cutting down the time it takes for real-time molecular sample analysis, studies need to be tailored to expand the scope of validation in order to be able to generalize the test results to real life clinical scenarios. Testing of the known as well as novel molecular biomarkers needs to be undertaken in a separate but more heterogenous and generalized population cohort with adequate power to truly test the validity of the findings, as is being done with HPV and *EGFR*. These can be independent retrospective, case-control and prospective study validations using carefully selected and matched large population-based cohorts, but need to be reserved for our most robust biomarkers that have been previously well characterized and optimized on multiple separate cohorts of convenience. Only then will we know the true potential of these biological markers as tools in molecular diagnosis of HNSCC and the full potential of the clinical applications of the results.

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