HHS Public Access

Author manuscript

Head Neck. Author manuscript; available in PMC 2015 June 02.

Published in final edited form as:

Head Neck. 2013 February ; 35(2): 294–306. doi:10.1002/hed.21975.

Opportunities and Challenges Facing Biomarker Development for Personalized Head and Neck Cancer Treatment

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Abstract

Head and neck oncologists have traditionally relied upon clinical tumor features and patient characteristics to guide care of individual patients. As surgical, radiotherapeutic, and systemic treatments have evolved to become more anatomically precise and mechanistically specific, the opportunity for improved cure and functional patient recovery has never been more promising for this historically debilitating cancer. However, personalized treatment must be accompanied by sophisticated patient selection to triage the application of advanced therapies towards ideal patient candidates. In this monograph, we review current progress, investigative themes, and key challenges facing head and neck cancer biomarker development intended to make personalized head and neck cancer treatment a clinical reality.

Keywords

Head and Neck Cancer; Biomarker; Personalized Therapy; Prognosis; Treatment Response; Radiotherapy; Functional Imaging; Positron-Emission Tomography; Magnetic Resonance Imaging; Human Papillomavirus; Epidermal Growth Factor Receptor; Microarray; Non-Coding RNA; DNA Damage Repair

INTRODUCTION

Head and neck cancer is a major international public health issue [(1)]. As now the fourth most common cancer worldwide, it afflicts more than 500,000 new patients each year and is a

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Disclosures: The authors report no relevant conflicts of interest.

major source of mortality in countries as diverse as India and France. While head and neck cancer is less common in North America, it continues to severely impact quality of life, productivity, and longevity. Current multidisciplinary treatment approaches are costly, complex, and morbid. Nonetheless, technical improvements in surgery [2] and radiotherapy [3] for locoregional management, and improved efficacy of systemic agents [4] have recently translated into tangible improvements in clinical outcomes [5]. Moreover, successful application of mechanistically-targeted biological therapy to head and neck cancer has been demonstrated [6, 7], and promises to further improve therapeutic ratio through individualized care [8].

Successful treatment individualization will rest heavily on successful patient selection, and will challenge our ability to accurately characterize tumor phenotype and host biology. Currently the only accepted means to communicate tumor behavior and prognosis is the American Joint Commission on Cancer (AJCC) staging system, which relies on clinical and radiological findings. The AJCC system has remained stubbornly consistent across published updates, and remains handicapped by deficiencies resulting in a predominance of patients being clumsily grouped together as “stage IV” despite heterogeneous presentations and prognosis [9-11].

A biomarker is defined as any biological characteristic with relevance to disease phenotype, or to the mechanism of action, target response, normal tissue toxicity, and/or clinical efficacy resulting from a specific intervention [12]. A validated, feasible assay platform must be available for objective, reproducible measurement. Biomarkers are classified according to incremental levels of utility (**Table 1**). A pharmacodynamic biomarker correlates with the direct mechanistic effect of an agent on tumor or normal tissue. A prognostic biomarker correlates with clinical outcomes independent of treatment. A predictive biomarker correlates with patient outcomes specific to a given treatment. Once validated by prospective clinical trial results, a biomarker may ultimately serve as a surrogate for clinical endpoints. Although most cancer biomarker research has focused on genetic or protein material obtained directly from tumor tissue, biomarkers can also be obtained from readily accessible host specimen sources, such as blood and saliva, or even non-invasively by functional molecular imaging. Ultimately, the intent of biomarker discovery is to provide objective tools for clinicians to detect cancer, confirm optimal treatment modalities, recognize treatment response and toxicity, and predict prognosis, all with accuracy, speed, and economy. Such biomarkers would provide obligate feedback information to guide selection of optimal targeted therapy to individual patients.

Incorporation of biomarkers into treatment of solid tumors is well established, exemplified by use of prostate specific antigen (PSA) for prostate cancer [13], hormone receptor and HER2-ERBB2 to direct systemic treatment of breast cancer [14, 15], and more recently the use of epidermal growth factor receptor (EGFR) and Ras mutational status to direct targeted therapy for colorectal and lung cancer [16, 17]. Nonetheless, progress in cancer biomarker development remains incremental. Formal incorporation of biomarker assessment can remain marginalized until the final phases of drug development due to stringent technical and financial challenges [18]. Regardless, biologically relevant disease characterization will be a prerequisite for the mechanism-driven cancer treatment paradigm to ultimately succeed

as hoped. In this review, we will discuss ongoing opportunities, challenges, and evolving research themes in the development of mechanistic biomarkers for head and neck cancer treatment.

TUMOR-BASED BIOMARKER DEVELOPMENT

Human Papillomavirus Infection—Human papillomavirus (HPV) is a DNA virus that infects skin and mucosal epithelium [19, 20]. It causes both benign and malignant tumors but can also exist in latent form, with no evidence of pathology. The HPV family contains more than 130 subtypes, each with tropism specific to a particular host site. Approximately a dozen high-risk subtypes are associated with urogenital and oropharyngeal malignancies contracted through sexual contact. HPV-16 is the dominant cause of oropharyngeal cancer (>90% cases), followed distantly by HPV-18 and HPV-33. The HPV genome encodes 8 proteins, labeled early (E) and late (L). E6 and E7 play important roles in HPV oncogenesis by interrupting key steps in normal cell-cycle regulation [21]. E6 expressed by high-risk HPV directs the ubiquitin-dependent degradation of p53, inhibiting critical tumor suppressor functions. E7 expressed by high-risk HPV binds the protein product of the retinoblastoma tumor suppressor gene (pRb) and promotes its degradation, releasing the E2F complex to stimulate cell cycle progression. E6 and E7 also impact additional downstream targets implicated in carcinogenesis, such as other members of the pRb pocket protein family, hTERT, and p21, but specific mechanistic roles for these actions remain unclear.

Recent epidemiologic studies have confirmed HPV-associated oropharyngeal cancer as a distinct head and neck cancer entity unassociated with classic risk factors (e.g. cigarette smoking and heavy alcohol intake) or secondary head and neck cancers [22]. Initial clues towards discovering this entity came from large American and international cancer registry databases demonstrating a rise in tonsil and base of tongue incidence in younger patient cohorts [23, 24]. Hammarstedt, et. al. evaluated 203 tonsil specimens collected between 1970 and 2002 for presence of HPV DNA and found an increasing incidence of HPV infection with each progressive decade [25]. More recently, D'Souza, et. al. confirmed a significant association between oropharyngeal cancer risk and life-time number of sexual partners [26]. The authors identified HPV-16 DNA in 72% of the orophayngeal tumor specimens. In separate studies, Smith, et. al. corroborated these findings [27] and also demonstrated a significant relationship between presence of HPV in exfoliated mucosal epithelial cells and risk of head and neck cancer [28]. While current reports demonstrate >50% prevalence of high-risk HPV infection in patients with oropharyngeal cancer, they also show a much lower association between infection and other head and neck subsites, such as oral cavity and larynx [29].

Recent studies have consistently demonstrated that patients with HPV-associated cancers have a significantly better prognosis than patients with HPV-negative disease. Within these studies, patients with HPV-negative disease had at least a 30% (absolute) lower overall and disease-free survival at 5 years. These dramatic differences exist despite the fact that HPV-associated cancers typically present with more advanced nodal stage. Licitra et al. evaluated 90 patients with oropharyngeal cancer treated with surgery [30]. HPV-positive patients enjoyed a significantly superior overall survival and tumor control rate. Fakhry et al.

evaluated HPV status in patients treated on a cooperative group phase II trial with induction chemotherapy followed by chemoradiotherapy for advanced stage laryngeal and oropharyngeal cancer. By multivariate analysis, presence of HPV infection predicted for better response to induction chemotherapy (82% vs 55%) and improved overall survival after a median follow up of 39 months (95% vs. 62%). Kumar et al. investigated HPV status of tumors in a cohort of oropharyngeal cancer patients undergoing chemoradiation [31]. This study found a clear association between HPV copy number and disease control. This could reflect the biological activity of one or more viral proteins, but might also help distinguish between active and latent infection. Most recently, Ang, et. al. retrospectively analyzed outcomes from a randomized cooperative group phase III trial testing alternative chemoradiotherapy regimens, and confirmed tumor HPV status to be an independent prognostic risk factor [32]. Patients with HPV-associated disease enjoyed a 58% relative reduction in mortality risk relative to HPV-negative patients.

HPV infection and p16 overexpression are mechanistically associated through the inactivation of Rb protein by viral oncoprotein E7 and subsequent upregulation of p16, a cell cycle dependent kinase inhibitor. Tumor cell overexpression of p16 can be detected by routine immunohistochemistry and can be leveraged as a surrogate for HPV-associated cancer [33, 34]. Although the data support the clinical utility of p16 testing, technical detection of both p16 and biologically relevant HPV infection remains a work in progress [35] and must be used in conjunction with cigarette smoking history in order to maintain prognostic significance [32, 36, 37].

Given the relatively good prognosis of HPV-associated oropharyngeal cancer, infection status has become a widely accepted prognostic biomarker and is being aggressively studied as means to improve clinical trial design and treatment selection. However, many questions remain. HPV infection status has not been formally validated as a predictive biomarker for any specific treatment modality or agent. Technical detection of infection remains non-standardized, and the relevance of infection in head and neck cancer outside the oropharynx is unestablished. Also, the exact relationship of HPV infection with other known biological pathways involved in head and neck cancer remains unclear. Continued HPV-specific biomarker discovery will be a priority in the coming years.

Epidermal Growth Factor Receptor Signaling Pathway—EGFR is a cell surface tyrosine kinase receptor critical to epithelial development and maintenance. Consistent with its central role in normal epithelial physiology, aberrant activation of EGFR signaling is associated with initiation and progression of a wide spectrum of epithelial cancers. EGFR has been identified in the laboratory [38, 39] and clinic [40, 41] as a prognostic biomarker and therapeutic target for head and neck squamous cell carcinoma. As a member of the ErbB receptor kinase family, EGFR signaling is closely tied to a number of key biologic pathways utilized by cancer cells to proliferate, invade, migrate, survive treatment, and foster supportive stromal angiogenesis, notably through downstream activation of the Ras/MAPK, PI3K/Akt, ERK, and Jak/STAT signaling pathways [42]. EGFR expression and activation is commonly elevated in up to 90% of head and neck carcinoma cells through transcriptional activation [43] or gene amplification [44, 45] of wild type gene product. Unlike carcinomas affecting the lower respiratory tract, activating mutations in the kinase domain of EGFR are

uncommon (0-7% frequency) and do not have identified functional significance in head and neck cancer [46, 47]. However, the constitutively active EGFRvIII deletion mutant has been observed in approximately 40% of examined cases [48].

Head and neck cancer enjoys the distinction of being the first human cancer site for which successful combination of selective EGFR inhibition with either cytotoxic chemotherapy [49-51] or radiotherapy [6, 52] has been demonstrated in randomized, multi-institutional clinical trials. The addition of cetuximab (a humanized monoclonal antibody specific to EGFR) to platinum chemotherapy significantly prolonged progression free survival in patients with late stage disease (5.6 vs. 3.3 months) [51]. In the case of definitive radiotherapy, a phase III trial demonstrated improved median locoregional disease control (24.4 vs. 14.9 months) and median overall survival (49 vs. 29.3 months) with the addition of concurrent cetuximab to definitive radiation [6, 52]. Small molecule inhibitors to EGFR also demonstrate modest (approximately 5% objective response rate) activity as single agent treatment of advanced disease [53]. While encouraging on the clinical front, published trials have failed to reproducibly identify any predictive biomarker to select patients for EGFR-targeted therapy. Although one study has suggested improved treatment response to accelerated radiotherapy in human tumors characterized as EGFR over-expressers by immunohistochemistry [54], a predictive association between quantified measurement of EGFR expression and survival following targeted therapy has not been validated.

Indirect detection of EGFR pathway activation, such as assaying EGFR gene copy number by fluorescence in situ hybridization, has been examined. Although some studies suggest poor prognosis following surgery or cytotoxic therapy in tumors with EGFR polysomy or gene amplification [44, 45], this has not been consistently reproduced [55, 56] and EGFR gene dosage has never been correlated with protein expression. A potentially more robust strategy would be to combine EGFR measures with mechanistically related markers of parallel or downstream signaling pathways, such as IGF1R, STAT3, and Src [57-61]. An important example of such an approach has been pilot correlation of EGFR expression with HPV infection status in clinical head and neck tumor specimens [62, 63]. These early studies have suggested that HPV infection is inversely correlated with EGFR protein expression, and that EGFR expression status may retain prognostic relevance regardless of HPV infection status. A more recent series has subsequently confirmed increased EGFR gene copy number status (as detected by FISH) to remain largely confined to HPV-unassociated (e.g. p16-negative) cancers [64]. Nonetheless, this study also showed that p16 expression supersedes EGFR-specific markers on multivariate analysis. Definitive prospective corroboration remains necessary, but taken together these findings suggest a potential need to regularly combine at least both HPV and EGFR specific biomarkers, if not others, to guide future clinical strategies.

Another approach to identify clinically relevant EGFR therapy-specific biomarkers would be to identify novel alternative mechanisms of EGFR promotion of head and neck cancer. As an example, recent reports indicate that EGFR and/or its downstream components have important functions after physical translocation to the nucleus [65]. Elevated levels of nuclear EGFR protein have, in fact, been associated with inferior radiotherapy outcomes in oropharyngeal cancer patients [66, 67], but specific downstream mechanisms of action remain

unclear. The continuing lack of clinically predictive EGFR-specific biomarkers in head and neck cancer, despite clear mechanistic role of EGFR activation in disease progression, serves as a humbling reminder of the complex array of resistance pathways available to tumor cells. Given the active use of EGFR inhibitors in clinical practice, priority must be given to identify EGFR-specific biomarkers for individualized treatment selection.

High-Throughput Biomarker Signature Identification—The limited success of individual markers, such as EGFR, to predict tumor behavior has led to a hypothesis that a "signature" of all detected molecular alterations in a tumor can more accurately define its phenotype. To broadly compile molecular changes within individual tumors, high-throughput techniques have been developed to investigate the full complement of RNA (microarrays) or protein (proteomics) expression alterations within a cancer or its surrounding microenvironment.

Microarrays permit global study of complete tumor transcriptomes. Head and neck tumor expression signatures have been compared with matched normal tissue, as well as metastatic disease, affording an opportunity to identify critical mechanistic pathways important for disease initiation and progression. For example, Chung, et. al. published a seminal study identifying four biological phenotypes of head and neck cancer defined by gene expression patterns [68]. This work was subsequently refined to identify upregulated epithelial-to-mesenchymal transition (EMT) and nuclear factor-kappaB-specific pathway expression in high-risk tumors destined to relapse [69]. Other studies have leveraged microarrays to compare functional differences between HPV-associated and HPV-unassociated disease [70], to confirm the clinical relevance of tumor hypoxia adaptation signaling [71], and to identify signaling pathway activation specific to metastatic disease progression to cervical lymph nodes [72]. The amount of data retrieved from microarrays can be overwhelming, with 300 to 825 genes frequently being identified as differentially expressed between tumor and control samples [73-76]. Because of the large amount of data generated, microarrays are mined for smaller sets of genes that can be used to stratify cancer phenotype. Methods for selecting these smaller sets of genes vary from agnostic selection of genes that are most over- or under-expressed by tumor cells, to investigator-defined gene candidates with specific biological functions relevant to the cancer phenotype under study [75, 77, 78]. The demands of large datasets have led to pilot attempts to utilize more sophisticated statistical analytic techniques to identify prognostic head and neck cancer signatures [79]. However, these techniques remain exploratory and large gene subsets (25 to 200 genes) are currently verified for predictive screening power in discrete training and validation tissue sets [75, 80], which mandates the availability of large independent collections of banked biospecimens. If identified within larger signatures, smaller predictive genes subsets (3 to 10 genes) can be further validated via direct interrogation of tumor tissue by quantitative PCR or immunohistochemistry (IHC); such smaller signatures are more potentially applicable for immediate translation to clinical trials. Several studies have correlated head and neck cancer gene expression with prognosis [71, 77, 78]. Subsequent studies have translated pilot microarray studies to accessible biofluids (blood and saliva) to screen for presence of subclinical disease [76, 81]. Thus, microarrays hold potential as screening tools and predictive biomarkers across a spectrum of accessible source materials. However, their current

contribution to head and neck cancer treatment remains limited to identification of candidate expression signatures requiring prospective validation through clinical trials.

Just as microarray platforms study genetic expression, proteomic techniques permit identification of downstream protein expression in tumors. Although there are many proteomic techniques, two have evolved into workhorses for translational cancer research. These are 1) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and 2) surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry. Distinct from microarray profiling, proteomic profiling does not identify specific proteins by name but instead recognizes the differential appearance or absence of protein peaks at specific mass/charge ratios. As for microarrays, the amount of data can be overwhelming, and many studies focus on protein peaks differing most between tumor and normal tissue. The pattern of these peaks can be successfully used to identify cancer versus normal mucosal tissue with up to 95% specificity and 94% sensitivity [82]. Proteomic techniques have been used to predict recurrence in small pilot studies [82, 83]. It will be important to validate the utility of proteomic signatures in prospective multi-institutional studies.

One of the challenges facing efforts to validate the clinical utility of proteomic analysis is that it requires fresh snap-frozen tissue, which is frequently unavailable from archival tissue banks. There has been progress using laser capture microdissection of formalin-fixed paraffin-embedded tissue for proteomic profiling [84]. However, the resource and throughput issues of laser capture make this less appealing than use of more readily accessible biofluids.

In summary, the above results confirm that emerging techniques for high-throughput signature identification hold the potential to mechanistically guide rational therapy for individual head and neck cancer patients. However, the field continues to face significant technical and logistical challenges. Neither the collection of samples nor any analytic platform has been standardized; as a result, identified signatures vary significantly from study to study. There are certain upregulated proteins, such as MMP1 [85], which are consistently identified across studies. These may indeed be eventually confirmed as clinically significant biomarkers. However, the potential use of high-throughput biomarker signatures to routinely triage cancer treatment strategies across different centers will not be fully realized until comprehensive standardization of biospecimen collection, microarray platforms, and proteomic techniques can be achieved.

BIOFLUID-BASED BIOMARKER DEVELOPMENT

Tissue samples are thought to be the most reliable source for biomarkers. Nonetheless, the invasive nature of biopsying has lead to investigation of easily accessible biofluids such as blood and saliva. Blood has the advantage of coming into contact with all parts of the body and providing a theoretical read-out for all tumor-related effects. The drawback is that proteins specific to disease are found in relatively low concentrations relative to abundant normal serum proteins such as albumin and immunoglobulins [86]. However, a number of studies, which will be discussed below, have been able to reproducibly detect circulating tumor proteins in serum.

Saliva is obtained completely non-invasively and comes into direct contact with head and neck mucosal cancers. Mucosal keratinocytes are normally shed into saliva [87], and may be joined by shed cancer cells. The disadvantage of saliva is that it continuously changes with eating, drinking, oral hygiene, smoking, and sleep/wake cycles [88]. Most well-controlled studies have collected saliva at a consistent time of day after a short interval of fasting [81, 89]. Another challenge with analyzing saliva is that the collection process has not been standardized. Some investigators have collected whole saliva in its natural state, while others have collected oral rinses using phosphate buffered saline or sodium chloride [85, 90-92], making comparisons between published series difficult and design of experiments for the uninitiated challenging. A recent study [85] highlights difficulties involved with analyzing saliva. Lallemant, et. al. studied salivary levels of MMP1, which is overexpressed by up to 500-fold in head and neck tumor samples relative to normal mucosa; nonetheless, these investigators found MMP-1 overexpression in the saliva of only 20% of sampled patients. Nonetheless, another study demonstrated matched DNA methylation in both tumor tissue and saliva in 96% of sampled patients [91]. Despite technical obstacles, an additional number of recent studies indicate that saliva remains a promising source of clinically relevant biomarkers [91-93].

Recent attempts have been made at analyzing the proteome in saliva [89, 90, 94], but the hostile physical environment of saliva has hampered progress [95]. More interest has been shown for proteomic analysis of plasma or serum. Attempts to identify individual serum protein prognostic markers or serum protein profiles to screen for early head and neck cancer have met with varying success [96-99]. One of the more successful serum proteomic profiling attempts is a recent study in occult papillary thyroid carcinoma patients which correctly identified patients harboring malignant disease with 95% sensitivity and 94% specificity [100]. A more recent study has suggested that mass spectroscopy analysis of serum or plasma can identify head and neck tumor cell dependence on EGFR signaling pathways and predict clinical response to targeted EGFR inhibition [101], while another has provided pilot evidence that serum cytokine and angiogenic factor profiles associated with tumor hypoxia can predict for response of head and neck cancers to induction chemotherapy [102].

EMERGING BIOMARKER STRATEGIES USING BIOSPECIMENS

DNA Damage Repair—Successful treatment of head and neck cancer with radiotherapy and chemotherapy requires creation of unsuccessfully repaired DNA damage within tumor cells. Delineation of biomarkers specific to the activity and integrity of tumor cell DNA damage repair networks is a promising area of interest. Several series have demonstrated expression of the repair protein ERCC1 to predict for chemotherapy response in esophageal [103], non-small cell lung [104], and head and neck cancer [105, 106]. Interestingly, one of these studies showed that ERCC1 overexpression in head and neck tumors was rare in non-smokers, suggesting a potential connection between DNA repair capacity and tumor HPV infection status [106]. Another DNA damage response protein associated with HPV pathogenesis, p53, is among the most extensively studied biomarkers in head and neck cancer. p53 mediates cellular sensitivity to genotoxic insult and suppresses tumor progression through a wide array of regulatory effects on DNA damage repair, cell cycle

control, apoptosis, and downstream gene expression [107, 108]. p53 mutations are associated with tobacco exposure [109] and have been observed in up to 80% of sampled head and neck cancers [110]. On a functional level, genetic inactivation of p53 can directly participate in the immortalization of cultured keratinocytes [111, 112]. Many institutional and cooperative group series have studied the prognostic significance of head and neck tumor p53 expression; however, disjointed use of heterogeneous technical methods across studies, coupled with the complex upstream regulation [113] and contradictory downstream functions [114] of the protein itself, have yielded inconclusive findings [115]. Nonetheless, more recent work has demonstrated that functionally disruptive p53 mutations may indeed predict for head and neck surgical outcomes [116]. Efficient genetic detection of tumor cells with p53 mutations in surgical margins is also feasible [117]; this approach is formally being studied in the cooperative group setting.

DNA damage repair-related biomarkers should intuitively predict for radiotherapy and chemoradiotherapy outcomes for head and neck cancer; regardless, none have been conclusively validated. Radiation causes many types of DNA damage, but the type of lesion most closely linked to target cell killing is the double strand break (DSB) [118]. A recent study has established Ku80, a mediator of DSB repair, as the first candidate DNA repair biomarker to show potential predictive value for head and neck radiotherapy [119]. There is mechanistic rationale to support an association between Ku80 expression and radioresistance. Ku80 is a key member of the non-homologous end joining pathway, the principal pathway used by healthy mammalian cells to repair DSBs [120]. In this series, Ku80 was overexpressed in half of tumors, and its expression was independent of all clinical and genetic covariates examined. Ku80 overexpression was an independent predictor for both locoregional failure and mortality following radiotherapy ($p < 0.01$). However, the predictive power of Ku80 overexpression was confined largely to HPV-negative disease, where it conferred a 9-fold greater risk of mortality at 2 years (**Figure 2**). Although Ku80 will require additional validation, this and other damage repair proteins hold tremendous promise as predictive markers with direct relevance to the mechanistic action of radiation-based treatment.

Tumor Hypoxia—Tumor hypoxia has long been associated with poor clinical outcome in head and neck cancer [121-123]. Tumor cell survivors of hypoxic stress are selected for reduced apoptotic potential, increased angiogenic signaling, enhanced metastatic capability, and greater resistance to radiotherapy. The biological cornerstone of hypoxia-specific stress responses is the hypoxia-inducible factor 1 (HIF-1) transcription factor [124]. Given HIF-1's pro-survival and angiogenic effects, targeted inhibition of HIF-1 signaling has generated interest as a therapeutic target for radiosensitization [125-127]. Tumor cell HIF-1 expression has been directly associated with inferior treatment outcomes for head and neck cancer [128-130]. Other hypoxia-associated proteins, such as the HIF-1 homolog HIF-2 [131], and downstream hypoxia adaptation proteins carbonic anhydrase IX [131, 132], lysyl oxidase [133], galectin-1 [134], and osteopontin [135] have been directly associated with poor head and neck radiotherapy outcomes in institutional and cooperative group trials. Additional promising factors associated with tumor cell adaptation to microenvironmental stress include overexpression of Src and E-cadherin [136], glioma-associated oncogene

family zinc finger 1 [137], uroporphyrinogen decarboxylase [138], as well as mutated TP53 tumor suppressor protein [116]. Interestingly, HPV E7 has recently been mechanistically linked to increased HIF-1 expression through inhibition of histone deacetylases [139]. Given the critical role these factors play in tumor progression and metastasis, continued investigation of hypoxic stress adaptation biomarkers remains a priority.

Micro-Ribonucleic Acid (miRNA)—As noted earlier, high-throughput evaluation of tumor mRNA expression is being actively pursued as a means to overcome the inherent limitations of individual biomarkers. However, there is growing recognition that mRNA-based tumor profiling is susceptible to complex post-transcriptional modulation, including regulation by non-coding miRNA. MiRNA are small transcripts 19-24 nucleotides in length which inhibit and target mRNA for degradation with varying degrees of specificity [140]. MiRNA have recently been shown to play important roles in human cancers through regulation of vital cellular processes such as proliferation, differentiation, and apoptosis [141, 142]. MiRNA expression profiles are tissue-specific and have been used to categorize cancer subtypes [143-145]. Early reports have demonstrated specific miRNA expression patterns unique to head and neck cancer which could potentially be used as a diagnostic or prognostic markers [146-149]. Mechanistic roles for specific miRNA have been suggested, most notably for *miR-21* which is overexpressed in human head and neck cancer tissue and promotes progression in head and neck tumor models [146, 150, 151]. Likewise, *miR-98* appears to regulate HMGA2-mediated head and neck tumor cell line sensitivity to chemotherapy [152], *miR-31* stimulates HIF pathway signaling in head and neck cell lines [153], *miR-221* promotes vascular invasion by oral carcinoma cancer cells [148], downregulation of *miR-375* is associated with increased carcinoma cell clonogenicity and proliferation [154], and *miR-26a* has been shown mechanistically to impair EZH2 oncogene-dependent cell growth and cell cycle progression in nasopharyngeal carcinoma cells [155]. Additional miRNA expression changes in head and neck cancer, including increased expression of *miR-423, miR-106b, miR-20a, miR-16* and downregulation of *miR-10a* have recently been described [154].

Despite this early progress, with the notable exception of *miR-21* and *miR-375*, there has been little reproducibility across published studies. MiRNA expression profiles have not been reproducibly associated with specific head and neck anatomic disease sites, nor with treatment outcomes [154]. Also, no associations between miRNA profiles and HPV tumor infection status have been reported to date. Early studies have suffered significantly from disorganized collection of tumor tissue (mostly from out-of-date institutional or cooperative group treatment trials), with only small subpopulations of patients with adequate tissue being available for miRNA PCR amplification and analysis. In addition, most studies have used limited amounts of normal tissue for baseline miRNA expression measurements, with many series not using matched normal control tissue obtained directly from study patients. This has unquestionably impacted the reproducibility, sensitivity, and clinical applicability of available published results. Just as with older high-throughput genetic and proteomic screening platforms, considerable work remains necessary to optimize and validate miRNA profiles as clinically meaningful biomarkers.

IMAGING-BASED BIOMARKER DEVELOPMENT

Tissue-based biomarkers, such as HPV and EGFR, promise to increase study power, reduce drug development costs, and limit pursuit of futile therapy [156]. Nonetheless, tumor tissue collection is expensive and complicated. Fresh tissue collection is difficult to obtain from patients referred to tertiary centers who have undergone outside workup and arrive with diagnostic pathology slides in hand. Development of disease-specific biomarkers collected from readily accessed biofluids (e.g. saliva, blood, urine) or tissue (e.g. buccal scrapings, skin) is one strategy to avoid such difficulties. However, such biomarkers currently rely on unproven or quickly evolving technical platforms and preclinical mechanistic data, and will likely remain exploratory for some time. An alternative exists which can leverage clinically validated technology and widely available expertise—non-invasive imaging-based biomarkers. The principle of this approach is straightforward, theoretically compelling, and exhaustively confirmed in animal models and human patients alike. Functional imaging can provide quantitative, non-disruptive, multiplexed characterization of whole tumor biology across all treatment and surveillance intervals without sampling deficiencies. Nonetheless, ongoing debate over competing techniques and targets, expensive upfront capital and long-term operational costs, and unresolved standardization of candidate imaging measures remain challenges to validation and wide scale deployment.

Fluorodeoxyglucose-Positive Emission Tomography (FDG-PET)—Anatomic imaging of head and neck cancer by CT or MRI has long been incorporated into the AJCC staging system. In contrast to traditional structural imaging information on tumor size and infiltration, functional imaging provides qualitative or quantitative snapshots of differential physiology within tumor and host tissues. FDG-PET, with or without registered CT imaging, has served as the functional imaging workhorse for head and neck cancer over the past two decades. FDG-PET/CT is most relevant to current care in the Western World; this technique spatially marries semi-quantified tissue glucose uptake data to contextual anatomic information provided by CT. Published data is extensive and has been summarized by meta-analyses [157], expert consensus reports [158, 159], and government-commissioned comparative effectiveness monographs [160]. Put simply, FDG-PET/CT incrementally improves disease staging accuracy and treatment response assessment over anatomic imaging [161-164], although potentially without compelling clinical benefit if performed unselectively [165-167].

Considerable interest has focused on FDG-PET/CT monitoring of disease response to radiotherapy. A number of groups have found that FDG-PET post-treatment restaging provides high negative predictive power [168-171]; accordingly, there is now growing acceptance of withholding consolidative neck dissection following radiotherapy in the absence of residual FDG-avid adenopathy [172], although others argue that expert clinical interpretation of serial CT imaging could achieve similar results [173, 174]. Our group's approach has been to emphasize identification of specific clinical situations where FDG-PET/CT diagnostic yield may be optimized. We have studied FDG-PET/CT utility in the context of other important clinical parameters, particularly HPV infection status, through a Bayesian, risk-based approach classically employed by clinicians choosing between alternative diagnostic tests in specific patients. We prospectively demonstrated that FDG-

PET/CT provides little value over CT alone in radiation response assessment for unselected patients with locally advanced HNSCC [166, 167]. Nonetheless, we found that FDG-PET/CT can significantly improve assessment of treatment response in high-risk patients, such as those with HPV-unassociated disease (**Figure 1**). Our results provide critical impetus to incorporate risk-stratification strategies into FDG-PET/CT assessment of radiotherapy response in locally advanced head and neck cancer. It is important to emphasize that such an individualized, context-specific approach will be relevant to any current or future imaging-based biomarker.

Early post-radiotherapy FDG-PET imaging of cervical neck nodes is frequently obscured by non-specific inflammatory changes, leading to decreased accuracy unless imaging is delayed following treatment completion [170, 175-177]. As a consequence, many patients cannot be efficiently triaged for appropriate upfront treatment intensity or towards timely salvage therapy. Ongoing development of novel ^{18}F-based radiotracers promises to expand the utility of PET/CT as an imaging biomarker through complementary mechanism-specific disease characterization [178, 179]. PET tracers with the most mature literature-based track record in head and neck cancer include the proliferation tracer 3'-deoxy-3'-^{18}F-fluorothymidine [180] and the hypoxia tracer ^{18}F-fluoromisonodazole (F-MISO) [181]. Given the well documented relationship between head and neck tumor hypoxia and aggressive phenotype, institutional series have generated particular interest in the use of FMISO-PET/CT for prediction and mechanistic characterization of treatment response [182, 183], as well as individualized targeting of hypoxic tumor subregions for escalated radiation dose delivery [184, 185]. Other biomarker candidates include the amino acid tracers *O*-(2-^{18}F-fluoroethyl)-L-tyrosine [186, 187] and L-3-^{18}F-fluoro-D-methyltyrosine [188], the dopamine precursor 3,4-dihydroxy-6-^{18}F-fluoro-L-phenylalanine [189] for head and neck neuroendocrine tumors, and the lipid biosynthesis precursors ^{18}F-fluoroacetate [190] and ^{18}F-fluorocholine [191].

Beyond PET/CT, novel vascular imaging techniques hold particular promise. Radiation stands apart from most systemic cytotoxic agents in its ability to impact tumor cells both directly and indirectly through destruction of supporting stromal blood vessels. Preclinical studies provide compelling data to show that radiation impacts tumor blood flow and vascular integrity, necessitating tumor adaptation and reconstitution of vessel function for regrowth [127, 192]. This indicates an opportunity to exploit vascular functional imaging for tumor response assessment. Early examples of potentially relevant clinical application include high-risk thyroid cancer, which is responsive to targeted anti-angiogenic agents [193] and nasopharyngeal cancer, which is currently being treated with combined radiation and anti-angiogenic therapy in the cooperative group trial setting (Radiation Therapy Oncology Group Study 0615).

Vascular Magnetic Resonance Imaging—Dynamic contrast enhanced-MRI (DCE-MRI) is a quantifiable, data-intense vascular imaging technique [194, 195]. It has been used in the preclinical setting to characterize whole tumor responses to radiation treatment, with or without sensitizers [127, 196-198]. DCE-MRI provides new opportunities to monitor tumor response through localization and quantitative measurement of changes in tumor perfusion and vascular integrity. DCE-MRI acquires T_1-weighted images before, during, and after

injection of an intravenous paramagnetic contrast agent, such as gadopentetate dimeglumine (Gd-DTPA). Two-compartment pharmacokinetic models are used to compute quantitative kinetic parameters, such as the volume transfer constant (K^{trans}) between blood plasma and extravascular-extracellular space (EES), the blood plasma volume fraction (v_p), and the EES volume fraction (v_e)[194]. A number of small institutional series have directly demonstrated the feasibility of DCE-MRI for evaluation of radiotherapy response [199-201], detection of recurrent disease [202], and pharmacokinetic analysis [203] in head and neck cancer patients. More recently, Cao, et. al. have demonstrated in a pilot experience of 14 patients treated for advanced head and neck cancer with chemoradiotherapy that DCE-MRI measures of blood volume within the primary tumor GTV obtained 2 weeks post-treatment can predict for local disease control [204]. Small patient numbers prevented meaningful analysis of nodal disease response.

Our group has prospectively imaged 14 patients with locally advanced oropharyngeal cancer with serial DCE-MRI performed at baseline, mid-treatment, and 6-8 weeks post-radiation treatment. Highlighting the relevance of imaging biomarkers to both disease response and toxicity prediction, we evaluated relationships between radiation dose and DCE-MRI parameter response in both tumor and normal salivary tissues. To achieve this, pixels within nodal target volumes and salivary gland regions of interest (ROIs) were deformably registered to IMRT dose maps via an “Demon's” image intensity-based algorithm [205] and binned into low, medium, and high dose groups (20-40 Gy, 40-60 Gy, and 60+ Gy, respectively). The low dose pixel group was of particular importance given that this represents gland subvolumes receiving doses close to known biological thresholds for salivary dysfunction following radiation treatment. Although K^{trans} values did not change significantly across treatment in salivary tissues at a group-wide level, we observed parotid ROI subvolumes receiving 20-40 Gy to dichotomously group towards increased or decreased K^{trans} changes by mid-treatment. Mid- and post-treatment v_e values increased significantly ($p < 0.02$) in both nodes and salivary tissues, with the magnitude of these changes suggesting dose response. Thus, serial K^{trans} measurements can potentially categorize at-risk parotid sub-volumes receiving biological threshold doses by tissue vascular responses at three weeks mid-treatment. Interestingly, v_e, an understudied DCE-MRI parameter, can potentially provide early and sustained quantifiable, dose-dependent measures of nodal and salivary gland response. Additional validation will be required and is ongoing.

Another promising vascular MRI technique with pilot experience in head and neck cancer is diffusion-weighted MRI (DW-MRI), which quantifies diffusion of water molecules with tumor tissue and may serve as a surrogate marker for treatment response [206]. Much like DCE-MRI, this technique is handicapped by limited published experience in patients, image analysis challenges, and high resource requirements. Nonetheless, encouraging pilot data suggests utility in head and neck cancer of DW-MRI for assessment of treatment response to radiotherapy [207], potentially as early as one week into treatment [208].

CONCLUSIONS

The search for predictive markers has longed shadowed the evolution of cancer medicine itself. Head and neck oncologists have traditionally leveraged clinical factors and general tumor features to guide their care. However, as treatments for head and neck cancer have become more precise and mechanistically informed, the opportunity for personalized application of selective treatments has increasingly relied upon effective patient selection. Such selection must be guided by validated prognostic and predictive biomarkers. Although progress outlined in this review demonstrates much promise, such as the growing importance of high-risk HPV infection as a means to guide treatment for oropharyngeal cancer, biomarker development still remains an unfulfilled, rate-limiting step towards capitalization of the promise of personalized cancer treatment.

Whether a biomarker is based on histology, imaging, or genomic information, its ultimate utility will require validated measurement that is faithfully reproduced across clinical settings and institutions. Towards this goal, there is increasing multi-disciplinary collaboration within and among academia and government agencies to guide [12] (and, in fact, ultimately regulate [209]) standardization of biomarker measurement. This is a particularly important need for functional molecular imaging [210], which has lagged in this area due to an exciting, but disjointed, proliferation of competing modalities and biological tracers. Looking forward, biomarker development promises to remain a critical determinant as to whether personalized care can reach its full potential for patients suffering from head and neck cancer.

Acknowledgements

Supported by the Hofstra North Shore-LIJ/Monter Cancer Center Grant Program and grants 1 F32 DC011446 and 1 R01 DC008579 from the National Institute on Deafness and Other Communication Disorders. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Deafness and Other Communication Disorders or the National Institutes of Health.

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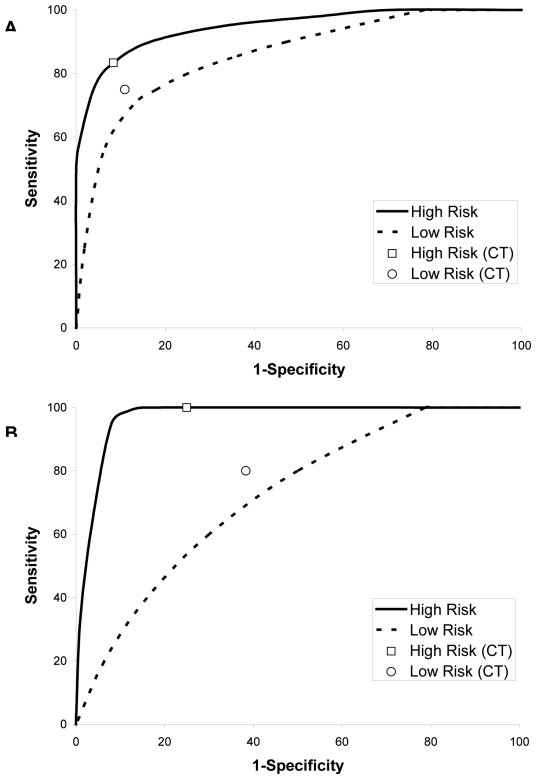

Figure 1.

Receiver operator curves for FDG-PET/CT are shown for primary (A) and nodal disease (B), with a solid line for high-risk patients and a dashed line for low-risk patients. For comparison, the sensitivity/specificity of CT is plotted for high-risk (open square) and low-risk (open circle) patients, as well.

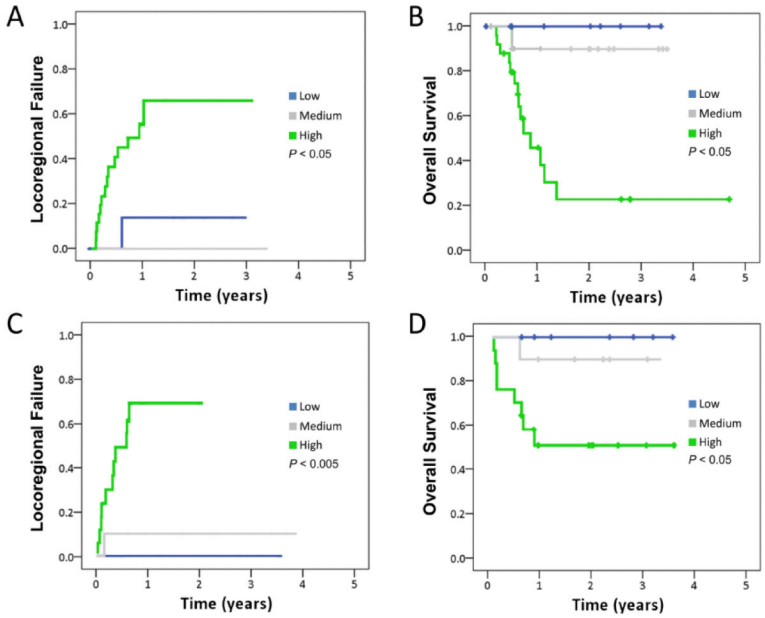

Figure 2.

Cumulative locoregional failure (A and C) and overall survival (B and D) rates for high-risk HPV-negative patients (A and B) and for a validation HPV-negative cohort (C and D) as a function of low, medium, or high Ku80 protein expression.

Table 1

Biomarker Types

Biomarker Type	Outcome Correlate	Specific Examples
Pharmacodynamic	Mechanistic treatment effect on tumor or normal tissue	mRNA/protein expression; protein phosphorylation; enzymatic activity; intra-tumor vascular function; normal tissue toxicity
Prognostic	Clinical outcome independent of treatment	High-risk HPV infection status in H&N cancer
Predictive	Clinical outcome specific to a treatment	Estrogen/progesterone receptor and HER2-ERBB2 expression in breast cancer; EGFR mutation status in non-small cell lung cancer
Surrogate Outcome	Direct substitution for clinical outcome	PSA in prostate cancer