

EXPERT INSIGHT

Digital pathology for the identification of prognostic biomarkers in head and neck cancer

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Head and neck cancers comprise a group of diseases with the most common being oral cavity and oropharyngeal cancers. The incidence of these cancers is on the rise but vary globally due to differences in risk factors such as alcohol, tobacco and betel quid consumption in addition to human papilloma virus infection. Despite advances in treatment, including cancer immunotherapy, the mortality rate remains high, which is mainly attributed to late diagnosis. Early detection of malignancies and prediction of malignant transformation in potentially malignant lesions are therefore vital to improve patient outcome. Digital pathology, which uses pre-defined algorithms to generate consistent and faster histopathological analysis, has made great strides in the quantification and identification of different markers capable of predicting disease progression, patient prognosis and response to therapy in head and neck cancer. The combination of digital pathology with different novel technologies including omics platforms, artificial intelligence and machine learning holds great translational potential for identifying prognostic biomarkers for head and neck cancer and beyond.

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HEAD & NECK CANCER... A QUICK OVERVIEW

Head and neck cancers comprise multiple types that affect different sites with two major subtypes being oral cavity cancer and oropharyngeal cancer. The incidence of oral cavity cancer is increasing globally with the steepest increase observed in the developing world [1], while human papillomavirus (HPV) driven oropharyngeal cancer is increasing in the developed world [1]. Changes and geographical variation in risk factors account for this rise, in particular, changes in alcohol, tobacco and betel quid consumption in addition to a major rise in HPV infections. The latter is worrisome as it is presenting at an earlier age group than what is traditional for these cancers and its prevalence is increasing independent of sex, race or ethnicity [2]. The incidence of HPV positive oropharyngeal cancers is increasing at an alarming rate leading to the suggestion that it is an 'emerging cancer epidemic' [3].

Despite advances in diagnosis and treatment modalities, the mortality rate remains high. In 2020, 377,713 new cases and 177,757 deaths of lip and oral cavity cancer were reported, in addition to 98,412 new cases and 48,143 deaths of oropharyngeal cancers [1]. The high mortality rate is mainly due to late diagnosis with many patients presenting at a stage where curative treatments are no longer an option. Therefore, screening tests and programs that can identify high risk patients at an early stage are essential.

Up until last year, it was thought that HPV driven oropharyngeal cancer could not be detected early and this was one of the reasons as to why there is no screening program for this cancer type. Tang *et al.* last year, for the first time detected 2 mm occult HPV driven oropharyngeal cancer in an asymptomatic individual, which paves the way forward for initiating a screening program for HPV driven oropharyngeal cancer [4,5].

Some head and neck cancers are preceded by potentially malignant lesions that carry a higher risk of developing into malignancy.

Screening for these lesions is also important to aid in the early detection of malignant transformation. Currently, there is a lack of biomarkers that can predict disease progression in these lesions and prognostic and predictive biomarkers to determine patient outcomes in an objective manner are also needed.

There are many attempts to introduce robust grading and classification systems for potentially malignant disorders to aid treatment planning and predicting disease progression. Assessed lesions were traditionally classified into mild, moderate and severe grades of epithelial dysplasia depending on the severity of cellular and tissue changes observed at a microscopic level. Although several iterations have been introduced to improve it, this classification system is not reproducible, has proven subjective, poorly transferable and unreliable in predicting malignant transformation [6–8]. There is, therefore, a move towards a simpler binary classification system that classifies epithelial dysplastic lesions into low- and high-grade dysplasia. Assessment of a binary system that classified dysplasia into low-risk and high risk lesions improved reproducibility, inter-pathologist agreement and was associated with 85% sensitivity and 80% specificity for predicting malignant transformation [9]. However, a reliable unified system that can accurately predict disease progression remains elusive.

CANCER IMMUNOTHERAPY IN HEAD & NECK CANCER

Cancer immunotherapy has proven successful in the management of many cancer types. There has been evidence of the potential for immunotherapy in head and neck cancer, however, there is a variable outcome in terms of response frequency and efficacy in patients. One of the main factors that lead to this variability is the type of tumor. While oral cancer has a significantly worse prognosis than oropharyngeal cancer, the latter's prognosis varies significantly depending on the HPV status, which is indirectly assessed based on

the overexpression of p16, where p16 positive (assumed HPV positive) oropharyngeal cancers have a better prognosis and respond better to therapy [10-12].

Immune checkpoint inhibitors have encountered success in the treatment of head and neck cancer and newer therapies are showing great promise [13]. Anti-PD-1 antibodies in particular have shown therapeutic efficacy. Pembrolizumab, was approved in 2016 following the KEYNOTE-012 Phase 1b clinical trial in which head and neck cancer patients with progressive disease following platinum-based therapy received pembrolizumab and showed a promising response with 18% of patients achieving an overall response with a duration of 12.2 months and overall survival of 13 months. These results compared favorably with cetuximab, an anti-epidermal growth factor receptor antibody [14]. Interestingly, pembrolizumab, demonstrated antitumor activity in both HPV positive and HPV negative oropharyngeal cancers [15]. A larger Phase 3 KEYNOTE-040 clinical trial compared pembrolizumab as a monotherapy to methotrexate, docetaxel, or cetuximab [16] and reported 19% improvement in overall survival in patients treated with pembrolizumab, although not significantly different from standard treatment [17]. Pembrolizumab as a monotherapy or in combination with chemotherapy (cisplatin or carboplatin) and 5-fluorouracil was compared with cetuximab with the same chemotherapy combination in the Phase 3 KEYNOTE-048 trial in recurrent or advanced metastatic head and neck cancer patients [18]. Data from the trial suggest that pembrolizumab as a monotherapy or in combination, significantly improved overall survival especially in PD-L1 positive tumors [19]. The National Institute of Health and Care Excellence (NICE) in UK has recommended the use of Pembrolizumab as a monotherapy as a treatment option for untreated metastatic or unresectable recurrent head and neck cancers that express PDL-1 [20].

Nivolumab was approved in 2016 as a result of the Phase 2 clinical trial (CheckMate-141) in which patients with recurrent

progressive post-platinum treatment head and neck cancer were treated with nivolumab as a monotherapy and once again, compared with methotrexate, docetaxel and cetuximab [21]. Nivolumab was associated with an improved overall survival and tumors that were positive for PDL-1 showed a better response to this anti-PD-1 antibody [22]. Interestingly, irrespective of the HPV status, patients with oropharyngeal squamous cell carcinoma responded better to nivolumab [22].

As oral potentially malignant disorders carry a higher risk of progression into malignancy, cancer immunotherapies are being assessed to treat these lesions before transforming into overt cancers. Targeting PD-1/PDL-1 in oral premalignant lesions was found to prevent their progression into malignancy in a murine oral squamous cell carcinoma model [23], suggesting the potential use of these immune checkpoint inhibitors in the treatment of oral potentially malignant disorders as well as established malignancies.

Despite being the first immune checkpoint inhibitors to be approved [24], antibodies targeting CTLA-4 did not meet the same success as those targeting PD-1 and PDL-1. As a consequence, there have been very few studies that looked into the potential of immune checkpoint inhibitors targeting CTLA-4 in head and neck cancer. A Phase 2 clinical trial that compared a combination of nivolumab and ipilimumab to nivolumab as a monotherapy in oral squamous cell carcinoma reported that patients in both arms of the trial showed evidence of response with the combination arm showing a marginally stronger response [25]. Furthermore, in a murine oral squamous cell carcinoma model, targeting PD-1 alone or in combination with CTLA-4 inhibition hindered the progression of oral premalignant lesions into malignancy [23].

However, the results of a more recent open label Phase 3 clinical trial that compared the anti-PDL-1 antibody (durvalumab) on its own or in combination with the anti-CTLA-4 antibody tremelimumab to the standard of care treatment (cetuximab, docetaxel, paclitaxel, methotrexate, 5-fluorouracil, TS-1, or

capecitabine) in recurrent or metastatic head and neck cancer patients did not find any improvement in overall survival [26].

Although anti-CTLA-4 antibodies received little attention in the treatment of head and neck cancer, there is abundant evidence that CTLA-4 plays an important role in the pathogenesis of these tumors. CTLA-4 genetic polymorphisms have been reported to be associated with susceptibility to head and neck cancer [27,28] and certain genotypes, such as CTLA-4 A/A at position +6230 A/G (CT60), were associated with poorer prognosis [29].

Head and neck cancers recruit regulatory CD4 T cells (Tregs) as an important immune escape mechanism. Despite the known suppressive role of Tregs in the tumor microenvironment, there are contradictions about their role in head and neck cancer [30]. Objective quantification and proteomic analysis of Tregs within the tumor microenvironment is essential to understand their role and identify important biomarkers on these suppressive cells. This is especially important as Tregs expressing high levels of CTLA-4 in head and neck cancer were reported to be associated with a high proliferative profile and were found to be highly suppressive [31] and the highest level of CTLA-4 expression in tumor infiltrating T cells in these tumors was observed in Tregs [32].

DIGITAL PATHOLOGY & BIOMARKER IDENTIFICATION & QUANTIFICATION

It has become apparent that identifying biomarkers that predict disease progression, patient prognosis or patient response to therapy as a stratification tool is essential for head and neck cancer patients.

Digital pathology has made great contributions in the quantification of various biomarkers including morphological, immunological and phenotypic markers that have enhanced our understanding of the pathogenies of different cancers.

Markers for detecting disease progression

Oral potentially malignant disorders carry a higher risk of malignant transformation. Therefore, identification of markers that can predict disease progression is essential to aid in the early detection of cancer formation and ultimately improving patient outcome.

Cell & tissue morphology

Changes in tissue and cellular morphology are important parameters in diagnosing potentially malignant head and neck lesions. Understanding these changes is essential to help identify markers for malignant transformation. To help improve the classification systems used for these lesions, image analysis was used to quantify various descriptors of tissue and cell architecture in normal, pre-malignant and malignant oral tissues. Fractal geometry quantified changes in the complexity of the basement membrane and different morphological parameters of cellular shape and size were objectively assessed to reveal gradual increase in basement membrane irregularity and changes in cell morphology associated with disease progression [33]. Our group is currently investigating the use of newer image analysis software packages to assess morphological changes in oral and oropharyngeal tissues in an attempt to identify markers associated with disease progression.

Angiogenesis

Changes in blood vessels are associated with disease progression where increased vascularization is observed in premalignant [34] and malignant oral lesions [35], suggesting their use for predicting disease progression [36]. Our group used digital pathology for the quantification of collagen IV, a marker of blood vessel basement membranes, and reported significant changes in the spatial distribution and morphometry of collagen IV expression in normal, premalignant and malignant oral [37] and oropharyngeal tissues with differences observed between HPV positive and negative

tumors [38]. Our findings highlighted the potential for using collagen IV as a marker for detecting disease progression and potentially a positive response to therapy given the role of blood vessels in drug delivery.

Immune markers

CTLA-4 has not received the same attention as PD-1/PDL-1. However, there is strong evidence of its involvement in head and neck cancer pathogenesis. Current anti-CTLA-4 antibodies can bind both the full-length receptor isoform and an alternatively spliced isoform, called soluble CTLA-4 (sCTLA-4) [39,40]. We have developed reagents that are selective for sCTLA-4 [41-43], allowing the study of the two forms of CTLA-4 in detail. We have recently shared our early data applying digital pathology in quantifying the expression of sCTLA-4 in normal, potentially malignant and malignant head and neck tissues. Our preliminary findings clearly show changes in the expression level and distribution of this immune checkpoint associated with disease progression suggesting sCTLA-4 as a promising marker for predicting disease progression in head and neck potentially malignant disorders [44].

Given the role that Tregs play in immune escape in head and neck cancers, digital pathology is a very useful tool in the quantification of these cells within the tumor microenvironment. Indeed, our group is currently using different image analysis techniques to assess Treg infiltration and distribution in normal, potentially malignant and malignant head and neck tissues to identify changes in these cells associated with disease progression.

Markers for predicting prognosis & response to therapy

Markers that can predict patient prognosis and response to therapy in head and neck cancer are needed to help stratify patients for different treatments, personalize therapies and improve patient outcome.

Clinical trials have shown that the response to anti-PD-1 therapy in head and neck cancer is stronger in PDL-1 positive tumors [19,22]. Furthermore, PDL-1 and PDL-2 expression was found to be positively correlated with Aldehyde dehydrogenase family 1 member A1 (ALDH1A1) expression with PDL-1 possibly involved in ALDH1A1 mediated poor prognosis [45]. Therefore, Quantifying PDL-1 expression is essential, and digital pathology and advanced image analysis techniques can provide robust and objective tools to assess the expression of this immune checkpoint.

Digital image analysis is also important for quantifying T cell infiltration in the tumor microenvironment including calculating the immunoscore as defined by CD3 and CD8 T cell infiltration in the tumor core and invasive margin in head and neck cancer [46]. Additionally, image segmentation algorithms were applied in multiplex immunohistochemistry in oropharyngeal cancer samples leading to better detection of different T cell subsets including, TH1-like and TH2-like TH17 T cells. TH2-like TH17 cells were more prominent in HPV negative cases and were spatially correlated with CD66b+ granulocytes suggesting a suppressive tumor environment [47].

Image analysis was also used to quantify tumor infiltrating lymphocytes within the epithelial compartment and in the stroma in head and neck cancer. This was coupled with the analysis of the immune cell infiltration based on RNAseq data and PD-L1 mRNA expression. Tumor infiltrating lymphocytes from hematoxylin and eosin-stained sections were found to be positive prognostic markers. Furthermore, sequencing data identified T cells as positive prognostic markers while PD-L1 was a negative prognostic marker [48]. Additionally, liquid biopsy-based techniques are coming to the forefront, as potential prognostic biomarkers. As an example, Kulasinghe *et al.* has shown that PDL-1 expression on circulating tumor cells can be used a potential biomarker to determine response to immunotherapy in a head and neck cancer patient [49].

Digital pathology & omics platforms

Technological advancements have enabled the incorporation of image analysis with proteomic and genomic analyses. The Hyperion™ imaging system for high dimensional proteomics analyses, which combines the simultaneous detection and quantification of over 40 markers with localization on histological sections, was used to quantify the tumor microenvironment in oral squamous cell carcinoma and highlighted the potential of this technology in predicting patient prognosis [50].

The NanoString GeoMx™ Digital Spatial Profiling technology, which combines image analysis with spatial genomic analyses, was used to analyze head and neck cancer samples from patients who received immune checkpoint inhibitor therapy and identified immune cell types and markers associated with disease progression [51]. In addition to analyzing immune cell types, these systems have a great potential in quantifying tumor mutation burden thus helping in the identification of therapeutic targets and predicting response to various therapies.

Digital pathology & artificial intelligence

Developments in artificial intelligence and machine learning have further revolutionized the applications of digital pathology.

Machine learning was used to create a model for predicting treatment in oropharyngeal squamous cell carcinoma while taking into consideration variables related to the tumors, socioeconomic, regional, and institutional factors [52]. Artificial intelligence is also used for Intensity-modulated radiation therapy (IMRT) treatment planning [53] and radiomics in head and neck cancer [54]. Furthermore, artificial intelligence was used to predict microsatellite instability and deficient DNA mismatch repair in hematoxylin and eosin stained colorectal cancer sections with high accuracy

in uniform datasets [55]. Artificial intelligence and machine learning have also been used in developing biomarkers for early detection of head and neck cancer by assessing metatranscriptomic data from saliva samples from normal, potentially malignant and malignant oral tissues [56].

The applications of digital pathology go beyond microscopic analyses of histopathological markers. Moderate associations have been found between PDL-1 expression and parameters of dynamic contrast enhanced MRI in head and neck cancer [57]. Weak associations were also observed between PDL-1 expression and diffusion-weighted imaging as quantified by apparent diffusion coefficient parameters [58]. This suggests the potential of applying artificial intelligence and machine learning at various levels including microscopy and clinical imaging to detect prognostic biomarkers that can predict disease progression and response to therapy.

Further studies that apply the power of machine learning and artificial intelligence are needed with the ultimate goal of improving patient outcome.

TRANSLATION INSIGHT

Digital pathology coupled with various technologies has great potential for identifying biomarkers that can help in detecting disease progression, predicting patient outcome and stratification of patients for specific treatments in head and neck cancer. Furthermore, these novel technologies have the potential to identify new targets for developing novel therapies based on quantifiable and objective assessment of cancer tissues.

As can be observed from this quick journey into the applications of digital pathology in head and neck cancer studies, the translational potential of these technologies in identifying diagnostic, prognostic and predictive biomarkers has become apparent, not only for head and neck cancer but for different malignancies and beyond.

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