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Oral Cancer and Oral Potentially Malignant Disorders

Guest Editors: Camile S. Farah, Sook-bin Woo, Rosnah Binti Zain, Alexandra Sklavounou, Michael J. McCullough, and Mark Lingen

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Editorial Board

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Editorial

Oral Cancer and Oral Potentially Malignant Disorders

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1. Introduction

Oral potentially malignant disorders (OPMDs) include a variety of lesions and conditions characterized by an increased risk for malignant transformation (MT) to oral squamous cell carcinoma (OSCC) [1]. Leukoplakia and erythroplakia are the most common OPMDs, while special emphasis has been placed on the premalignant nature of oral lichen planus (OLP) [2].

It is generally accepted that the histopathological features of a given lesion, especially the presence and degree of epithelial dysplasia, are currently the most useful indicators of MT risk [3]. However, histopathological assessment alone does not provide an accurate assessment of MT risk, and other features, such as clinical and molecular parameters, must be taken into account. In this regard, the clinical characteristics of OPMDs can show considerable variation within the same histopathologically defined entity that may be critical to the likelihood of progression towards malignancy, thus, serving as prognostic factors of MT and facilitating clinical decisions for further intervention and followup.

Currently, leukoplakia is defined by the World Health Organization as "a white plaque of questionable risk having excluded other known diseases or disorders that carry no risk" [4]. It is a clinical term only and histopathologically may be defined variously from atrophy, hyperplasia, to dysplasia. All frictional disorders (such as chronic cheek biting or benign alveolar ridge keratoses) are excluded by this definition. Two main clinical variants of leukoplakia are recognized: homogeneous leukoplakia with a low risk of MT and nonhomogeneous leukoplakia with a higher risk of MT [5]. The latter can be further subclassified into speckled leukoplakia (red and white but predominantly white), erythroleukoplakia (red and white but probably not predominantly white), nodular leukoplakia, verrucous leukoplakia, and proliferative verrucous leukoplakia [4].

The frequency of epithelial dysplasia, carcinoma-in-situ, or invasive SCC in leukoplakias varies from 8.6% to 60.0% [6–9]. MT of epithelial dysplasia or carcinoma-in-situ occurs in 13.6% to 36.4% of cases [6, 10], and the annual MT rate has been variably reported from 1 to 3% for all leukoplakia [6, 7, 10, 11]. It is well accepted that nonhomogeneous leukoplakia is associated with a higher risk (4- to 7-fold) for MT compared to homogeneous lesions [1–3]. The presence of an erythematous component (erythroleukoplakia) seems to convey a greater risk for MT. This is in agreement with the high malignant potential of pure red lesions (erythroplakia), which, despite its low prevalence ranging between 0.01% and 0.2%, is associated with a very high MT rate which approximates 55–65% in some studies [12]. Furthermore, the frequency of epithelial dysplasia, carcinoma-in-situ, or invasive SCC in erythroplakia is greater than 90% at first biopsy [12]. In addition, proliferative verrucous leukoplakia (PVL), a distinct entity with multiple verruciform white plaques

showing a relentless tendency to expand and recur and a predilection to affect nonsmokers and especially women around 50–60 years, has been linked to a MT rate that may eventually approximate 100% [13].

Recently, optical diagnostic aids have been used to better define the clinical features of OPMD and to provide some insight into underlying cellular and molecular changes occurring in these lesions, as highlighted in the paper by Bhatia et al. in this special issue. Light-based devices at various wavelengths have been explored and show promise in assisting the clinician to detect and better visualize OPMD and oral cancer. Surgeons can also use this technology for assessment of tumour margins during surgical resection [14]. During surgical removal of malignancies, surgeons usually remove approximately 10 mm or more of normal appearing mucosal margins with the hope of achieving a margin clearance of 5 mm or more to compensate for fixation shrinkage of the formalin fixed resected specimens. Such clearance has been the routine standard used by surgeons in attempting to prevent recurrence from marginal areas with occult changes. There is thus a dependence on the pathologists' interpretation of surgical close and clear margins which have been used as predictors of tumour recurrence and survival. Despite this, there is still a high recurrence of primary tumours (up to 25%) which may result from the inability to correctly predict the molecular changes already occurring in these margins. This highlights the need to further explore adjunctive methods such as autofluorescence and narrow band imaging in a manner similar to that used for detection of OPMDs. The paper by Diajil et al. in the current issue adds credence to this approach, since laser excision of OPMD as determined by normal operatory light inspection resulted in a significant number of recurrences at the local site, and clinical resolution was most commonly seen with small and intermediate lesions compared to larger sized lesions. Furthermore, as outlined by Kudo et al. in the current issue, histology-based 3D reconstruction of serial tissue sections for evaluating tumour architecture has potential to better inform our understanding of cell invasion at the deep invasive front.

Other than the importance of clinical subtyping of OPMD, the malignant potential of oral leukoplakia and erythroplakia appears to be affected by other parameters, such as site and size. The lateral border of the tongue and the floor of mouth have been correlated with the highest percentage for MT (as high as 44% and 24%, resp.) [1–3]. Despite the limited available data on the prognostic significance of the size of OPMDs, it appears that larger lesions (i.e., greater than 200 mm^2) are associated with a higher risk (up to 5.4-fold) of MT [1].

Despite the aforementioned MT rates for leukoplakia, it has been known for some time now that so-called "benign hyperkeratosis" transforms to OSCC. As early as 1987, Silverman et al. [6] in the United States noted that 37 out of 235 cases of "benign hyperkeratosis" transformed to invasive carcinoma. Subsequently, Schepman et al. [7] from The Netherlands noted that MT occurred in 6 out of 20 (30%) cases of nondysplastic leukoplakia. Holmstrup et al. [15] in Denmark noted that 2% and 11% of patients with untreated or treated nondysplastic leukoplakia, respectively, developed invasive carcinoma and more recently in 2007 Hsue et al. [10] from Taiwan noted a MT rate of 3.6% in their cases although many of their patients also had submucous fibrosis from the use of betel quid.

Several questions come to mind in this regard. (1) How can benign hyperkeratotic lesions transform to carcinoma? (2) Is there true "benign hyperkeratosis"? (3) Are there any features clinically or molecularly that can help to distinguish between "true" benign reactive keratosis that has no MT potential and nonreactive keratosis that does have a potential for MT? (4) If nondysplastic leukoplakia undergoes MT in at least 4% of patients, does this change the long term management of these patients?

2. How Can Benign Hyperkeratotic Lesions Transform to Carcinoma?

There are several factors to consider. Firstly, the diagnosis of dysplasia is notoriously difficult with only, at best, moderate interexaminer agreement between pathologists [16]. As one would expect the discordance is the largest in cases of mild dysplasia and less so in moderate and severe dysplasia. The epithelial changes in mild dysplasia may be subtle or focal and may be attributed to epithelial changes secondary to reaction to injury or inflammation, often termed "reactive epithelial atypia." This can be observed in biopsies of oral lichen planus, or at the edge of an ulcer. Conversely, cases of reactive epithelial atypia may be overdiagnosed as dysplasia. This is why some pathologists have moved to using a binary system for the diagnosis of dysplasia—low-grade versus high-grade with high-grade dysplasia purportedly more likely to transform to invasive cancer [17]. There is an understanding that low-grade lesions or mild dysplasia may be difficult to distinguish from reactive atypia. Furthermore, architectural abnormalities of dysplasia, such as verrucous configuration without evidence of cytologic dysplasia, are just as important in the evaluation of dysplasia.

Secondly, a single biopsy from a large or nonhomogenous clinical lesion may not be representative. In the study by Lee et al. that evaluated 200 cases, underdiagnosis from a single biopsy versus multiple biopsies was 29.5% and 11.9%, respectively [18]. The prevalence of invasive carcinoma in the resection specimen was 12.0% versus 2.4% in the single versus multiple biopsy cases. This raises another important issue. If within a large or nonhomogenous leukoplakia there are areas that show dysplasia and other regions that show only hyperkeratosis without dysplasia, are the areas of hyperkeratosis without dysplasia precursors to dysplasia?

Thirdly, it may be that leukoplakic areas that show keratosis or hyperkeratosis histopathologically but have little evidence of cytologic abnormality may in fact represent the very earliest changes in carcinogenesis. In two clinically recognised entities, verrucous leukoplakia and proliferative verrucous leukoplakia, the histopathologic changes are those of hyperkeratosis and verrucous epithelial architecture, with only minimal or no evidence of epithelial dysplasia. Proliferative verrucous leukoplakia is a clinicopathologic entity first

recognized by Hansen at al. in 1985 [19]. They noted cases of leukoplakia that were "slow-growing, persistent, irreversible, and frequently developed erythematous components." Studies have subsequently shown that 40–70% of such lesions will develop invasive carcinoma when followed over time [13]. It is very likely that all three factors play a role to a lesser or greater extent, in the transformation of so-called "benign hyperkeratosis" to invasive carcinoma.

3. Is There True "Benign Hyperkeratosis"?

Many pathologists use the diagnostic phrase "hyperkeratosis, acanthosis (benign epithelial hyperplasia)" to encompass both frictional keratoses and true leukoplakias without epithelial dysplasia. Lesions of chronic frictional keratosis from parafunctional habits (cheek biting or chewing) and benign alveolar ridge keratoses, common on the retromolar pad, all represent frictional keratoses and will also exhibit hyperkeratosis and acanthosis [20, 21]. As such, when a clinician receives a report of "hyperkeratosis, acanthosis, or epithelial hyperplasia," without further comment, the lesion could represent an entirely benign lesion caused by friction or a true leukoplakia with the potential of developing dysplasia or invasive cancer. This will confound the results when such lesions are used in leukoplakia research, not as controls, but as lesions of true leukoplakia, possibly early or mild dysplasia. Indeed, many publications have used just such lesions of frictional hyperkeratosis with epithelial hyperplasia under the diagnosis of "leukoplakia."

4. Are There Any Features Clinically or Molecularly That Can Help to Distinguish between "True" Benign Reactive Keratosis That Have No MT Potential and Nonreactive Keratosis That Do Have a Potential for MT?

Clinically, leukoplakias (in particular, homogenous leukoplakias) are for the most part demarcated plaques with a sharp border between the keratotic area and the adjacent normal mucosa, at least for part of the lesion. Although this feature is not present in 100% of cases, it is present in most cases of homogenous leukoplakia and less so in erythroleukoplakia. Homogenous leukoplakia also tends to show shallow fissuring on the surface. At a molecular level, studies have shown a variety of genetic changes, none of which have been consistently noted to be present in dysplasia [22]. It is unclear whether this is due to frictional or reactive keratoses with mild atypia being included in cases of dysplasia.

5. If Nondysplastic Leukoplakia Undergoes MT in at Least 4% of Patients, Does This Change the Long Term Management of These Patients?

Presently, there is no consensus regarding the management of leukoplakia, with some favoring a "watch-and-wait" attitude towards dysplasia while others favoring excision [9, 23–25]. Patients with "benign hyperkeratosis" are generally managed with a "watch-and-wait" protocol. One argument made against removal is the high rate of recurrence of leukoplakias. However, if cases of frictional and reactive keratoses are incorrectly included in the group of leukoplakic lesions studied, a high recurrence rate is inevitable. Another argument against removal is that some dysplasia (especially mild dysplasia) regresses. The counter-argument to this is that some cases diagnosed as mild dysplasia may actually represent reactive epithelial atypia from trauma such that regression and recurrence would be expected. Removal of true leukoplakias with a narrow margin with the understanding that a recurrence may occur necessitating a wider excision of the subsequent smaller lesion may be less expensive than repeated biopsies over many years. The followup is also easier since the decision after complete removal is a binary one: absent or present versus is it larger or slightly morphologically altered?

With all these points considered, it is vitally important then to recognize that accurate diagnosis of leukoplakia can only be made when the clinician and pathologist work together and share information.

The malignant potential of OLP, a relatively common, chronic, immunologically-mediated, inflammatory disease has been the subject of significant controversy with conflicting data and opposing views [26–31]. It has been suggested that several reported cases of OLP progressing to OSCC may in fact represent oral erythroleukoplakias with an associated lichenoid inflammatory reaction (so-called "lichenoid dysplasia"). This is particularly noted if such lesions are unilateral and located only at a high risk site such as ventral tongue, but where the histopathology showed a "lichenoid" lymphocytic band [32]. Another explanation is that there is coexistence of two separate processes such as a leukoplakia that develops within typical oral lichen planus. Further, it should be noted that a lymphocytic response to dysplasia and tumor is one of the hallmarks of malignancy [33–35], and can confound the histopathological assessment of these lesions. Nonetheless, most authorities agree today that actual OLP is associated with a relatively low risk for malignant progression.

A large number of studies report that progression to OSCC is more common in the erosive/ulcerative and atrophic OLP, the so-called "red forms", compared to the "white forms." An increased risk for MT has also been suggested for the hypertrophic (or plaque-type) form of OLP; the issue with a diagnosis of a pure plaque-form of OLP is how this is different from a leukoplakia. However, the possibility of MT exists in all forms of the disease. Moreover, changes in OLP clinical presentation and severity over time are frequent (e.g., transition from reticular to erosive and/or hypertrophic forms), underlining the need for adequate followup of all cases. It should also be stressed that oral lichenoid lesions, such as those attributed to allergic contact reaction to amalgam or other dental materials and those related to drug-induced allergic reactions, show considerable overlap in their clinical and histopathological features with OLP and may also be linked to an increased likelihood of MT [32].

The frequency of MT in OLP has been reported to range from 0.4 to 5.3% in various studies, usually not exceeding 1% [26–31]. This relatively broad range can be attributed to various factors such as differences in sample size, diagnostic criteria both clinically and histopathologically, follow-up time, assessment of other risk factors, and applied therapeutic interventions. In general, the factors underlining the possible malignant potential of OLP and the significance of related parameters, such as exposure to known carcinogens, remain largely unknown. Recent insights into molecular aberrations in OLP lend further support to the premalignant nature of this condition, which may be related to the effects of chronic inflammatory stimulation. Expression of p-Akt, p-mTOR, and phospho-pS6 has been demonstrated in a subset of OLP cases as outlined by Prodromidis et al. in this special issue, suggesting that activation of Akt/mTOR/pS6 may occur in the context of OLP, possibly contributing to the premalignant potential of individual cases.

Despite the availability of a significant number of cohort studies with an adequate number of patients and an appropriate followup, the exact prognostic significance of the clinical features of OPMDs is not fully appreciated. Large, well-designed multicenter prospective studies with appropriate inclusion and diagnostic criteria and long term followup are in order. Importantly, photographic documentation should be integral to the process. It may be that we need to revisit the classification of OPMD with a view to dividing lesions into "high risk" (leukoplakia and submucous fibrosis) and "moderate-to-low risk" subtypes (OLP, nicotinic stomatitis from reverse smoking, and lupus erythematosus).

OSCC in the oral cavity is a devastating disease and most arise from preexisting leukoplakia. Although clinicians may still struggle to make a decision as to which lesions might become malignant, once these lesions progress to malignancy, the clinical diagnosis of these as oral cancer is, for the most part, reasonably uncomplicated. More than 90% of oral cancers are OSCC and the histopathological diagnosis is also fairly straightforward. The areas of concern would then revolve around identification of microinvasion and superficially invasive SCC. The group of noninvasive verruca-papillary lesions, termed by some as "verrucous hyperplasia" histopathologically and "verrucous leukoplakia" clinically, have also been recognized as a type of OPMD [36]. Controversies exist as to whether some of these verruca-papillary lesions are already malignant in nature, as explored in the paper by Kallarakkal et al. in the current issue.

What is becoming clearer though is the importance of stratifying tumors within the broad family of cancers known as "head and neck malignancies" and "oral cancer." It is now perfectly clear that this group of tumors is a heterogeneous cluster. It is necessary to pay close attention to this when designing molecular classification profiles. The difficulty in achieving a molecular classification that can be fully utilized for all head and neck cancers is due to the extreme heterogeneity in the genetic expression of these cancers [37]. This marked heterogeneity of their genetic profile is partly due to the fact that head and neck cancers consist of a mixture of cancers of the oral cavity, oropharynx, nasopharynx, hypopharynx, and larynx, known to have varying aetiological factors. Oral cavity cancers are more heterogeneous than other head and neck cancers, and this may be related to different risk habits associated with varied subsites, namely, lip, buccal mucosa and alveolus, and tongue and palatal mucosa. In addition, gene expression microarray studies have found distinct differences in the gene expression profile of oral cancer patients with betel quid chewing habit compared to patients who smoke tobacco [38]. More recently, detailed analysis of HPV positive and negative head and neck squamous cell carcinoma genomes highlights the presence of two distinct groups based on HPV positivity [39] and emphasized the findings of Agrawal et al. [40] and Stransky et al. [41]. High-throughput molecular technologies are currently being used in an effort to further delineate the molecular pathways of cancer and to help with substratification of this large group of heterogeneous tumours that we still term "oral cancer" [42, 43].

As we come to terms with the clinical and histopathological features of OPMD and oral cancer, their molecular features are still largely elusive despite a dramatic increase in the quantity of research being undertaken in this field. Currently the only FDA approved targeted agent for treatment of patients with advanced head and neck cancer is cetuximab (an EGFR-directed monoclonal antibody). Biomarker studies utilising different approaches and samples have been undertaken, and these are highlighted in this special issue by John et al., Prasad & McCullough, and Brooks et al. To advance this research, there is an urgent need for worldwide groups to form research consortia to allow pooling of research materials so as to allow adequate sample sizes to validate current findings and subsequently develop accurate, validated instruments to improve the management and outcomes for oral cancer patients. This type of effort would certainly drive the advancement of therapeutic aspects for head and neck cancers, which currently lags behind cancers from other sites such as breast, colorectal, and lung. With readily available and less expensive molecular diagnostics, a collaborative effort and clarity of terminology and classification, we may unravel the mystery of oral cancer and OPMD and learn more about the sequence of genomic alterations that occur to firstly cause the appearance of a keratosis with minimal or no dysplasia, mild (low-grade) dysplasia, moderate and severe (high-grade) dysplasia, carcinoma-in-situ, and finally invasive carcinoma. This enhanced understanding of the disease process will allow for easier and earlier recognition of MT, better intervention and decreased morbidity and mortality, and ultimately better management for our patients.

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Review Article

Chemokines and Cytokines as Salivary Biomarkers for the Early Diagnosis of Oral Cancer

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Chemokines have been shown to be important in both inflammation and carcinogenesis and are able to be measured in saliva with relatively robust methods including enzyme-linked immunosorbent assays (ELISA). Thus it has been hypothesized that patients with oral cancer and oral potentially malignant lesions will have elevated levels of specific chemokines in oral fluids and that this may be used as a marker of both the early detection of malignant disease and progression to malignancy. The concept that salivary biomarkers can be easily measured and indicate disease states has profound consequences for clinical practice and may open up new strategies for the diagnosis, prognosis, and potential therapy of oral squamous cell carcinoma (OSCC). This review focuses on our understanding of cytokines and chemokines and the potential role that they may have in clinical practice.

1. Introduction

Oral cancer is the eleventh most prevalent cancer worldwide [1]. Oral cancers in Australia account for approximately 2-3% of all cancers and approximately 1% of all cancer deaths, with an increasing incidence over the past decades [2]. The most common oral cancer is oral squamous cell carcinoma (OSCC), which makes up 90% of all oral cancers [3], and if diagnosed early has a five-year survival rate of around 85% [4]. However, the early phase of oral cancer is often asymptomatic. Mortality for oral cancer is high because most patients seek care only when they experience late-stage symptoms (pain, persistent ulceration, unexplained bleeding, or an oral or neck mass), at which stage the disease is advanced and the survival rate decreases as low as 15–50%. Early detection of oral cancer is therefore paramount for improving survival rates and prognosis for patients with the disease.

Current diagnostic techniques focus on detection of malignant and potentially premalignant lesions in the oral cavity. Early lesions may present as unhealing lesions, mucosal colour changes, pain, tenderness or numbness, protuberances, or rough, thickened, crusted, or eroded areas [5]. Typically, premalignant and malignant lesions begin as a subtle red or white patch (erythroplakia or leukoplakia) that eventually ulcerates and progresses to an exophytic mass [6]. Regular comprehensive examinations of the oral cavity form the backbone of oral cancer screening and are especially critical in patients with identified risk habits and factors such as tobacco smoking, excessive alcohol consumption, and human papilloma virus infection [7].

The advantage of the standard visual and tactile examination is that it is simple to perform and requires no added equipment. However, subtle lesions may pass undetected, and it is difficult to make a visual distinction between benign, premalignant, and malignant lesions. Adjunctive techniques have been developed in recent years to facilitate making this distinction and enhance the effectiveness of oral examinations. Techniques such as vital staining (Toluidine Blue) and visualisation adjuncts (VELscope and ViziLite) highlight abnormal mucosa by targeting tissues undergoing rapid cell division and areas of high metabolic turnover [8]. Another adjunctive technique employs transepithelial sampling of the oral mucosa for cytologic analysis (OralCDx Brush Test system). While promising, these emergent technologies have yet to reproduce the sensitivity and specificity of examination via tissue biopsy and histopathological examination, which remains the gold standard for oral cancer diagnosis [8].

A new focus of research is the use of salivary diagnostics for early detection of OSCC, which have the advantage of being noninvasive and nontoxic. Proteins, mRNA, enzymes, and chemicals extracted from saliva have been found at sufficiently distinct levels between OSCC and control samples to be considered as potential biomarkers [9]. These biomarkers could be important indicators of physiological or pathological states and provide information for the detection of early and differential markers for disease. Salivary biomarkers offer an easy, inexpensive, safe, and noninvasive approach for disease detection [10]. They have the potential to serve as a widely available screening tool that does not rely on the localization of a lesion for diagnosis [11]. This advantage over other detection methods gives salivary biomarker screening the potential to identify patients with malignant and potentially malignant lesions.

Recent studies have assessed variation in biomarkers in patients with oral cancer. Using an array of biomarkers from oral rinses from 40 HNSCC patients and 39 controls assessed by ELISA assays, it has been shown that it is possible to distinguish HNSCC cases from controls, particularly when the patients demographics were also considered [12]. Further, extensive analyses of the plasma levels of 48 proteins (26 cytokines, 10 chemokines, and 12 growth factors) in 111 untreated OSCC patients, 112 healthy individuals, and 107 individuals with potentially malignant oral mucosal lesions showed that the levels of 12 proteins were significantly dysregulated in OSCC patients serum [13]. Furthermore, a recent extensive study had been undertaken to substantiate the development of salivary biomarkers. This study assessed a panel of putative OSCC markers in 395 subjects in 5 independent validation cohorts and found them to be independently validated, reproducible, and robust for use in a reference laboratory [14]. Thus, such studies indicate the potential of specific deregulated proteins as predictive biomarkers in oral cancer. This review will discuss the potential of cytokines and chemokines as salivary biomarkers for the early diagnosis of oral squamous cell carcinoma.

2. Cytokines

Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cell membrane cytokine receptors can induce a number of activities within the cell, such as growth, differentiation, or death [15]. Most cytokines have pleiotropic effects; however, some are generally considered as proinflammatory, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukin-1beta (IL-1β) [16–19], whereas others are associated with anti-inflammatory effects, such as transforming growth factor-beta-1 (TGF-β1) [20].

Over the last 5 years, a considerable effort has been undertaken to analyse the salivary proteome. A large number of nonredundant proteins have been recognised in saliva, with one study [21] reporting over 1400 and another [22] almost 2,000, reflecting the diversity of salivary biomarker profiles that may identify and potentially aid in the management of a range of diseases [23].

Of particular interest has been the use of salivary cytokine levels as markers of both cell proliferation and oral cancer [24]. The most studied cytokines include epidermal growth factor (EGF), interleukins-6 and -8, vascular endothelial growth factor (VEGF), interleukins-4 and -10, tumour necrosis factor (TNF) and endothelin [24, 25].

Several studies have assessed interleukin-6 (IL-6), a multifunctional cytokine that participates in the inflammatory and immune responses and has been shown to promote the growth of cancer cells as well as associated with an increased rate of metastasis and an altered immune status [26–32]. Interestingly, IL-6 would appear to have different effects on different cell populations, stimulatory for some cell types while inhibitory for others [30]. IL-6 can promote tumor cell proliferation in several tumor cell lines, including human cervical carcinomas mediated cachexia [33]. In contrast, another study indicated that expression of IL-6 and its receptor can be inhibitory for cell proliferation and is correlated with good prognoses for patients with breast cancer [34]. IL-6 also has a demonstrable direct effect on cancer cells via inactivation of the p53 tumour suppressor gene as seen in human multiple myeloma cell line KAS 6/1. IL-6R overexpression was associated with larger tumors and more advanced histologic grade [35]. Irrespective of the role of IL-6, there is increasing evidence to support higher levels of IL-6 in the saliva of patients with oral cancer, as well as oral potentially malignant lesions, than in normal controls [36]. In a recent trial of 29 consecutive patients being treated for oral cancer, it was shown that patients had much higher salivary concentration of IL-6 than controls and that this concentration increased during the treatment period returning to baseline levels at discharge [36].

Other studies however have assessed a panel of pro-inflammatory cytokines as markers of malignancy [27, 37]. A recent study assessing the levels of IL-1α, IL-6, IL-8, VEGF-α, and TNF-α in saliva, measured using quantitative ELISA, was undertaken in a group of 18 patients with tongue SCC [38]. These salivary biomarkers were demonstrated to be increased in patients with oral cancer; significantly increased in a subgroup of patients with endophytic tongue cancer and IL-8 levels; particularly shown to correlate with poor prognosis; and intriguingly also found to be higher in control individuals who both smoked and consumed alcohol daily [38].

However, it should be noted that elevated levels of IL-6 and IL-8 have also been detected in other studies in the saliva of patients with periodontitis [39, 40]. The main limitation of this study is the relatively small sample size ($n = 10$); nevertheless, although IL-8 was found to be higher in patients with periodontitis than in healthy controls, it is detected at significantly much greater levels in patients with OSCC [41]. Yet if this is true, it should be possible to differentiate between an inflammatory process and a neoplastic process by the amount of IL-6 and IL-8. The study in 2008 by Arellano-Garcia et al. has multiple important innovative aspects, including the fact that it showed that multiplex bead based assays were as effective as ELISA assays for quantification of proteins in saliva and that IL-8 and IL-1beta were expressed at significantly higher levels in OSCC patients [41]. Although

there were only 20 cancer patients, with 20 age and gender matched controls, this study nevertheless clearly indicated the potential of constituents of saliva as biomarkers for oral cancer.

A further study assessing salivary levels of TNF-α, IL-1α, IL-6, and IL-8 in a group of nine patients with OSCC with matched healthy controls [42] attempted to assess the relative influence of periodontal inflammation by using a modified gingival index to matched patients and control samples [42]. It found that IL-6 was statistically significantly higher in patients with OSCC. Interestingly though, several patients were edentulous, and thus neither they nor their matched controls would help discriminate the role of periodontal inflammation in relative salivary chemokine level, a fact compounded by the small sample size (9 patients with OSCC) [42].

Thus far then, the results of a number of studies would indicate that salivary cytokine levels are very likely to provide useful information of the presence of disease, epithelial behaviour, the local inflammatory response, and carcinogenesis. However, larger sample sized studies are required to investigate salivary cytokines and their role in the diagnosis of PML and OSCC while at the same time being able to deal with the obvious local confounding factor of inflammation and in particular periodontal disease. Further studies of the potential of a panel of salivary cytokines as a screening tool for oral cancer are apparently ongoing, the results of which are eagerly awaited as this is likely to have a profound impact on the early detection of oral cancer and thus morbidity and mortality [11]. The complexity of undertaking such a study, that would require a large number of patients who have oral cancer, patients with potentially malignant mucosal disease, and sufficient health controls as well as patients with non-neoplastic mucosal disease. This comprehensive study, thoroughly analysing the diversity of these salivary biomarkers present in health and disease, is at the same time both daunting and necessary.

3. Chemokines

Chemokines are a superfamily of structurally related cytokines, which share an ability to chemotactically attract their target cells along a concentration gradient [43]. It is through this ability that these molecules play an integral role in the migration of immune cells to areas of pathogen challenge. Chemokines also mediate the movement of specific cells involved in inflammatory responses that subsequently result in cellular interactions critical for mounting immune responses [43].

All chemokines are small proteins, ranging in weight from 6 to 14 KDa. There are now over 50 identified chemokines and 20 chemokine receptors [44]. Chemokines and chemokine receptors can be classified into 4 main structural families, dependent upon the position of the cysteine residues near the N-terminus. These families are the CC, CXC, C, and CX3C, with the X denoting the number of amino acids between the cysteine residues [45, 46].

Chemokines are secreted in response to signals such as proinflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF), and interferon-c (IFN-c) and thus they play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes [47].

Once induced, the directed migration of cells expressing the appropriate chemokine receptors occurs along a chemical ligand gradient known as the chemokine gradient. This allows cells to move toward high local concentrations of chemokines [48]. Chemokines induce chemotaxis through the activation of G-protein-coupled receptors (GPCRs), subsequently involving adhesion molecules and glycosaminoglycans (GAGs) [49]. Chemokines bind to specific cell surface transmembrane receptors coupled with heterotrimeric G proteins, whose activation leads to the activation of intracellular signaling cascades that prompt migration toward the chemokine source (chemotaxis) [50]. This interaction results in multiple signal transduction pathways being activated. One of the characteristics associated with the chemokine system is its redundancy. It has been shown that a single ligand can bind to multiple receptors and in turn a chemokine receptor may bind multiple ligands [51]. Also, many of the inflammatory chemokines have wide target cell selectivity, with some acting both on the cells of the innate and adaptive immunity [51].

The function of chemokines can be subdivided into two main families: those that are induced after inflammatory stimuli, the inflammatory chemokines, and those produced constitutively in tissues, the homing chemokines [52]. In addition to their roles in the immune system, chemokines and chemokine receptors are also involved in the pathology of a number of diseases, such as infections (e.g., HIV-1/AIDS), autoimmune disorders (e.g., psoriasis, rheumatoid arthritis, and multiple sclerosis), pulmonary diseases (asthma and chronic obstructive pulmonary disease), transplant rejection, cancer, and vascular disease [53]. Furthermore, there would appear to be significant overlap between chemokines as some of the inflammatory chemokines appear to be produced constitutively in some areas of the body [54] and some of the chemokines designated as homing chemokines can be upregulated by inflammatory stimuli [55]. For example, LARC/MIP-3α plays a role in both homeostatic trafficking of leukocytes, as well acting as an inflammatory chemokine during host defense [56].

Although the detection of chemokine levels by enzyme-linked immunosorbent assay (ELISA) has become a sensitive and specific method to determine the chemokine profile in patient fluids, this is not able to fully represent the actual inflammatory conditions *in vivo*. Indeed, many chemokines are posttranslationally modified by proteolytic cleavage, which can render an agonist more active or inactive or even convert the active chemokine into a receptor antagonist of the intact molecule [57].

Nevertheless, using ELISA, a recent study assessed the saliva of patients with oral cancer for the presence of both inflammatory chemokines (CXCL8, CXCL10, and CCL2), homeostatic chemokines (CXCL4, CCL14, and CCL18) [58]. Further, individuals with and without periodontitis were used as controls and it was found that H&N carcinomas give rise to a change in the chemokine composition of the oral fluid with a significant increase in CXCL8, CXCL10, and

CCL14 before therapy, a finding that was not reproduced after therapy [58]. However, the levels detectable by ELISA were very low and it is likely that more refined methods could indicate not only intact chemokines, but also those modified posttranslationally [58]. These authors conclude that it can be expected that specific truncated chemokines, as well as the proteases involved in this truncation, will be linked to particular disease states. These authors postulate that proteomic analysis of biological fluids will further our understanding of the pathogenesis of specific diseases and provide solutions for new diagnostic and treatment options [58]. Since chemokines in disease can be occasionally involved in excessive recruitment of inflammatory cells, prevention of this recruitment may be an effective anti-inflammatory strategy. Furthermore, chemokine receptors are intimately involved in cellular recruitment and, along with CD4, have been shown to be an essential cofactor enabling HIV-1 viruses to infect cells [59]. Thus, in diseases that have a profound effect on the immune system, such as infection with HIV, there are various points of potential intervention that could provide anti-inflammatory and anti-HIV infectivity therapeutics, including prevention of the receptor-ligand interaction, prevention of the chemokine-glycosaminoglycan interaction, interfering with the signaling pathways that are induced upon receptor activation, and modification of receptor trafficking pathways [59].

However, these postulated potential interventions need considerable further study as the apparent redundancy in the expression of chemokines, and the overlap between homeostatic and inflammatory chemokines pathways, makes them difficult targets for diagnosis of diseases and therapeutics. In addition, it has been shown that the enhancement of the inflammatory response is aided by synergistic activity of chemokines for leukocyte migration [60]. Hence, blockage of a single chemokine may downregulate other immune responses, because of the inhibitory effect on its synergy with other chemokines [60]. Thus, there is a need to refine our ability to assess the presence of chemokines in disease states, the presence and specificity of chemokine receptors, and the specificity of functional active chemokines in specific disease states, prior to being able to define selective and specific targets for treatment.

Significant change in our understanding of the role of chemokines in OSCC has occurred in a fairly short time. A relatively early study assessed the presence of a particular chemokine (CXCL12) and its specific receptor (CXCR4), revealing that the receptor was more prevalent in oral cancers that metastasized, suggesting that this chemokine/receptor may be important in the regulation of tumour growth and organ-specific lymphatic spread [61]. A further, extensive study of 85 patients with oral SCCC utilized immunohistochemistry, RT-PCR, and western blot to assess the expression of a different chemokine, CCR7 and its ligand CCL21, in 85 patients with oral squamous cell carcinoma [10]. It was shown that CCR7 expression was positively correlated with lymph node metastasis, tumour size, and clinical stage, and these authors postulated that the interaction between this chemokine and its receptor may be significant for the induction of lymphatic spread.

The mechanism by which chemokines and chemokine receptors are involved in oral carcinogenesis has been extensively studied [19, 62–64]. CCL5 (previously known as RANTES—Regulated on Activation, Normal T cell Expressed and Secreted) has been shown to play a crucial role in migration and metastasis in human cancer cell lines and further showed that that CCL5/CCR5 axis enhanced migration of oral cancer cells, probably via MMP-9 [65]. An extensive investigation of 253 oral cancer patients, matched with 347 controls, the presence of mutations (single nucleotide polymorphisms) in the genes of specific chemokine ligands and receptors (CCL5 and CCR5) revealed an interesting dichotomy of the presence of mutations increasing risk for oral cancer while at the same time raising the potential that oral cancers with a specific chemokine profile may well have enhanced protection from metastases [66]. In a bid to rectify the dysregulated CC chemokine receptor (CCR5)/ligand, a recent study used interferon-α2b (IFN-α2b), known to upregulate CCR5 expression [67], in a small cohort of 12 oral cancer patients. These investigators showed that enhanced T-cell-mediated tumor cell killing upon IFN-α2b treatment and they postulate that this immunotherapy treatment may be combined with standard chemotherapy for better clinical outcome [67].

The SDF-1/CXCR4 (stromal cell derived factor 1/chemokine (C-X-C motif) receptor 4) pathway has been suggested to play a role in the metastatic dissemination of neoplasms with migration toward SDF-1 by tumor cells bearing CXCR4. Mutation in the gene of a specific chemokine receptor (CXCR4) has been noted to have an increased likelihood of more advanced oral cancer (stage III and IV by 2.66-fold) [68]. A study of 71 patients with HNSCC assessing the tissue expression levels of SDF-1 and CXCR4 found that patients with low SDF-1 had poorer metastasis-free survival ($P = 0.026$), disease-free survival ($P = 0.006$) and overall survival rates ($P = 0.002$) [69]. A recent immunohistochemical study has confirmed that this relationship showing a significant relationship between CXCL12 and CXCR4 was found both in potentially malignant lesions and oral cancer [70]. An *in vitro* experiment has recently shown that, with synthetic biology approaches, signalling selective inhibition of the CXCR4 prevented the metastatic spread of neoplastic cells [71].

Previously, investigations based on the known association between the chemokine ligand CXCL13 and prognosis of oral cancer found that the chemokine ligand/receptor axis of CXCL13/CXCR5 is not only important for cancer bone invasion and metastasis but may also be a potential therapeutic target to prevent OSCC bone invasion/osteolysis [72].

4. Conclusion

Current research has identified deregulated cytokines in OSCC as well as oral potentially malignant lesions, robust and reproducible methods for the assessment of these cytokines in saliva, and the possibility of a rapid salivary test as an indicator of disease and risk of malignancy. Ongoing development of such a method will have profound impact on oral cancer screening and the early diagnosis of oral cancer,

potentially resulting in early treatment and a decrease in the high levels of morbidity and mortality associated with OSCC. Furthermore, there are indications that it may be possible to utilize our enhanced understanding of the chemokines associated with disease progression, metastases, and bone invasion to develop novel methods for the treatment of oral cancer.

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Review Article

MicroRNAs in Head and Neck Cancer

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microRNAs (miRs) are small noncoding single-stranded RNAs, about 19–25 nucleotides long. They have been shown to be capable of altering mRNA expression; thus some are oncogenic or tumour suppressive in nature and are regulated by cellular and epigenetic factors. The molecular pathogenic pathway of many cancers has been modified since the discovery of miRs. Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer in the world, has recently been associated with infection by the human papillomavirus (HPV). miR expression profiles are altered in the transition from dysplasia to carcinoma, with some changes being specific to the underlying risk factor. This difference is particularly significant in HPV-positive HNSCC where host miRs are modulated by the virus, creating a different profile to HPV-negative HNSCC. Saliva, as an easily collected proximal biofluid containing numerous miRs, presents an attractive noninvasive diagnostic tool in detecting HNSCC and determining prognosis. Furthermore, miRs may play a role in the analysis of surgical margins for residual tumour extension and in the development of novel miR-based therapeutic targets and agents.

1. Introduction

Head and neck cancer (HNC) is the 6th most common cancer in the world [1], referring to cancers of the aerodigestive tract, including lip, oral cavity, nasal cavity, paranasal sinuses, pharynx, larynx, oropharynx, hypopharynx, salivary glands, and local lymph nodes [2]. 90% of all HNCs are squamous cell carcinomas (HNSCCs), arising from the mucosal lining in these regions [2]. 80–90% of these are related to prolonged alcohol and tobacco use, while 30–50% have been associated with the human papillomavirus (HPV) [3], with type 16 being the most common type detected in HNSCC [2]. There is significant geographic variation in its incidence, with South-East Asia, the Pacific regions, Latin America, and parts of Central and Eastern Europe presenting a higher incidence than other regions; for instance, HNSCC is the most common cancer type in India, accounting for 40% of all malignancies [4, 5]. The 5-year survival rate of smoking associated with HNSCC is still 30–50%, with survivors experiencing poor quality of life [3]. Furthermore, HNSCC is usually not detected in the early stages of the disease as they may not display clinical symptoms [3, 6]. Hence methods for early detection and diagnosis of lesions with malignant potential [7–9], measures for prevention, and novel treatment methods are instrumental for improving treatment outcomes and patients' quality of life.

miRs are small, single-stranded RNA molecules that were first discovered in 1993, shown to influence the larval development of the nematode *Caenorhabditis elegans* by regulating translation through an antisense RNA-RNA interaction [6, 10, 11]. In June 2013, the miRBAse database recorded 1600 *Homo sapiens* miRs [12]. Although they are noncoding, they are considered to influence the expression of many protein-coding genes in the human genome [3]. miRs are associated with mRNA translation and degradation, influencing organ development, cell differentiation, proliferation, apoptosis, and stress responses [10, 13–15]. Multiple miRs can target one mRNA, while one miR can influence mRNA transcripts of numerous genes [3], implicating them in tumour development by modulation of cellular levels of specific oncogenes or tumour suppressor genes [10, 16].

miRs are transcribed by RNA polymerase II (RNA Pol II), producing primary miRs (pri-miRs) which are converted to precursor miRs (pre-miRs) by Drosha (an RNAase III

endonuclease) and DiGeorge syndrome critical region gene 8 (DGCR8) [10, 17]. pre-miRs (70–100 nt long) are transported to the cytoplasm by exportin 5 and processed by Dicer (an RNAase III enzyme) and TRBP (a Dicer partner) to form double-stranded (ds) RNA, approximately 22 nt long [10, 17]. This dsRNA consists of mature miR and the complementary strand (miR*) [10]. miRs* are usually degraded but may also be functional [10]. Mature miRs can modulate the translation of protein-coding mRNAs by base pairing to partially complementary regions [10]. It recognizes specific sequences, and, using Argonaute 2 (Ago2), TRBP, and RNA-inducing silencing complex (RISC; a multiprotein complex responsible for site-specific cleavage of the target mRNA), it degrades mRNA repressing its translation [10, 17]. miRs have also been found to interfere with RNA binding functions (decoy activity) in a RISC-independent manner [10, 16, 18].

miR expression is regulated at the transcriptional and posttranscriptional levels, by cellular factors (including c-Myc (oncogenic protein inducing oncomir expression), p53 (tumour suppressor protein inducing tumour suppressor miRs), and E2F) or defects in miR biogenesis machinery (Drosha, DGCR8, exportin 5, Dicer, TRBP, and Ago2) [16, 19]. Changes in miR expression in cancer may be due to changes in activation of gene transcription regulators at the promotor [16, 20] (e.g., HIF-1α [21] and PKCα [22]), epigenetic regulation by altered DNA methylation or histone deacetylase inhibition resulting in reduced or lost expression [16, 23], single nucleotide polymorphisms (SNPs) in pri- and pre-miRs as well as in miR biogenesis pathway genes [24–26], loss or gain of chromosomal material since miR encoding genes are commonly located in fragile regions associated with oral SCC (OSCC) [16, 27–29], or deregulation of key genes involved in miR biogenesis [30].

Despite the transformation rate of potentially malignant oral lesions to OSCC (31.4%) [31], clinical and histological characteristics have limited potential as predictors of transformation and do not aid in early diagnosis of HNSCC [32]. It has been shown that as many as 50% of HNSCCs may arise from apparently clinically normal mucosa, thus posing an inherent diagnostic challenge [33]. Although it is established that potentially malignant oral lesions (with the most common being leukoplakia and erythroplakia) and epithelial dysplasia are statistically more likely to progress to cancer, the actual mechanisms are poorly understood, and it is not inevitable that a dysplastic lesion will progress to cancer [33]. Thus upon clinical diagnosis of HNSCC, disease staging is often advanced, and prognosis is poor [34, 35]. The histopathological interpretation of tissue biopsies can be subjective and is thus prone to a considerable range of interpretation [35]. Similarly, no definitive, validated criteria exist for predicting which dysplastic lesions are most likely to progress to cancer over time [33, 36]. Given the current state of scientific knowledge, the presence of dysplasia can only be used to indicate that an oral lesion may have an increased risk of malignant transformation [37]. In spite of the considerable advances in diagnostic tools and treatment over the past three decades, mortality of HNSCC patients continues to be high, and current treatment modalities are still associated with many adverse effects and decreased quality of life [38].

2. Utilizing miRs in Early Diagnosis of HNSCC

Overexpression of oncomirs in tumours contributes to oncogenesis by inhibiting tumour suppressor genes and/or genes that control cell differentiation or apoptosis. For example, the *p53* gene is inhibited by miR-372/373. Other known oncomirs are miR-17-92, miR-21, and miR-155 [3, 19, 32, 39]. Some miRs have oncogenic properties on deregulation [15, 40]. For example, upregulation of mir-21 leads to inhibition of apoptosis [41], while its downregulation leads to chemoresistance [42]. Conversely, miRs may be characterized as tumour suppressors if their normal action opposes oncogenesis (e.g., let-7, and miR-15a/16-1, miR-34a, miR-143/145) [19, 43].

miRs are tissue specific and bind to the 3′ untranslated (3′-UTR) region of their target mRNA(s), resulting in a profound control over gene expression at the posttranscriptional level [6, 13, 14, 19, 44]. Significantly, some miRs may be oncogenic in one cell or tissue type but tumour-suppressive in another, depending on the tissue context and target genes [19]. Consequently specific miR patterns may be used to distinguish cancer cells from normal cells, as well as to identify the tissue of origin in carcinomas with unknown primary tumour, forming a means to early diagnosis of HNSCC [14, 43, 45, 46]. It has been suggested that specific miRs can be used as diagnostic markers for HNSCC diagnosis, such as overexpression of miR-21 and -205 [15, 47]. Avissar et al. [48] have claimed a sensitivity of 92% and specificity of 93% in classifying HNSCC using the expression ratio of miR-221 to miR-375. Furthermore, miRs can also discriminate between subtypes of a specific type of cancer and precise oncogenic abnormalities [16].

miRs may also be used as diagnostic indicators of metastatic disease [15]. The hallmarks of cancer progression have been described as self-sufficiency in growth signals, ignorance of antigrowth signals, apoptosis evasion, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [49]. Figure 1 shows the deregulated miRs in each stage of cancer progression, from normal epithelium to metastatic cancer [3]. Barker et al. [50] found that miR profiles were distinct and specific for HNSCC in the tonsil, base of tongue, and postnasal space. Furthermore, the authors established that miR expression profiles between primary cancer and its nodal metastatic disease were consistent with the implication that miR profiles may be used as a diagnostic tool to determine whether the nodal metastasis is from the oral cavity, particularly when the primary tumour cannot be identified—a significant advantage of miR profiling [15, 16, 50]. Conversely, Hui et al. [51] did not find distinct expression profiles between the three subsites investigated—the hypopharynx, oropharynx, and larynx. This conflicting data may be due to technical problems as well as differences in stage, grading, and sampling from multiple anatomical sites but signifies the need for further analysis [40].

miRs further have a prognostic importance in determining the survival of patients with HNSCC [15]. For example, it has been suggested that high expression of mir-21 can be used as an independent predictor of poor survival for patients with tonsillar SCC and a significantly lowered 5-year survival in patients with HNSCC [41, 52], while lowered miR-205

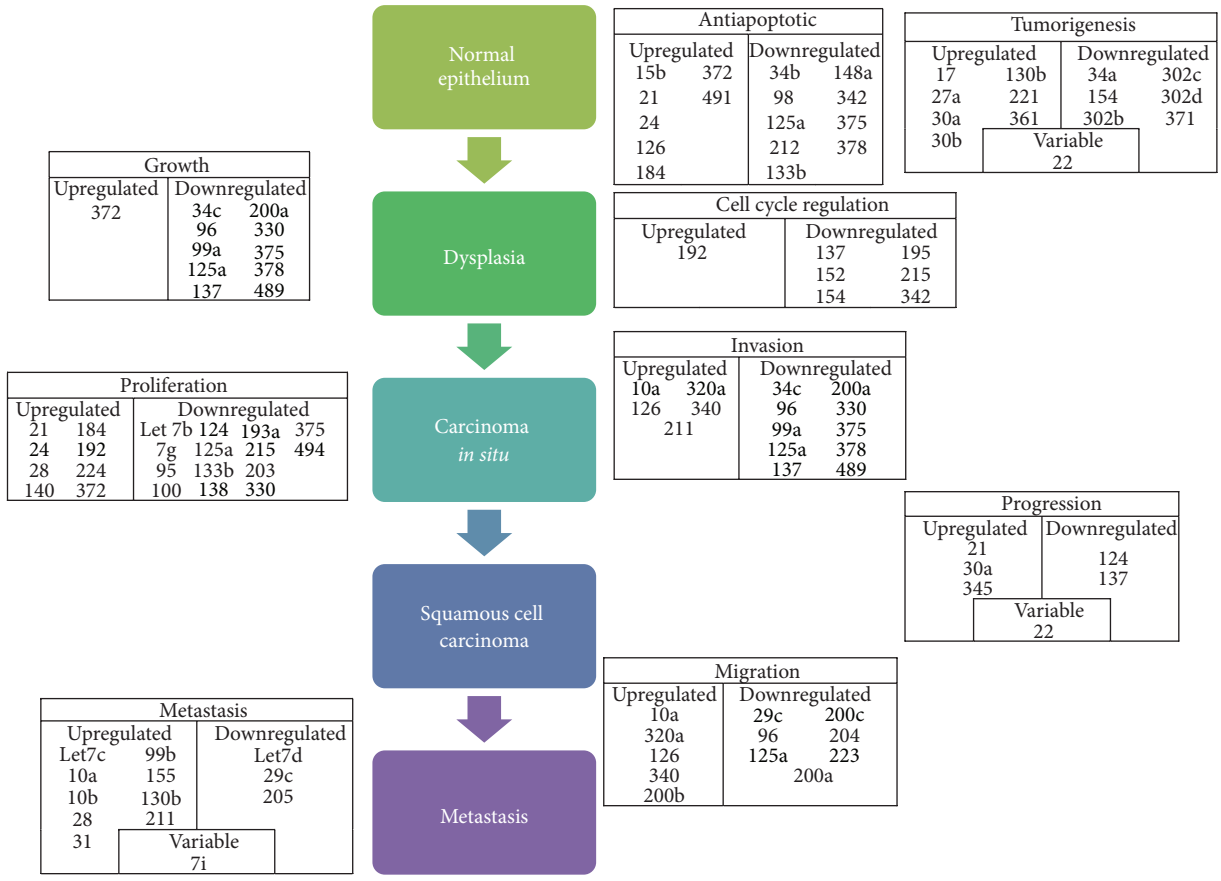

Figure 1: The role of miRs in regulating the transformation of normal squamous epithelial cells into carcinoma cells, ultimately resulting in metastasis (adapted from [3]).

expression [53] and increased miR-451 expression [51] have been significantly correlated with locoregional relapse of HNSCC irrespective of disease severity at diagnosis and treatment.

miRs may also be used as biomarkers in bodily fluids such as blood and saliva, since they have been shown to circulate stably in both healthy and cancer patients [15, 16]. This stability is due to their inclusion in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles, or exosomes, which prevent their degradation by RNAses [3]. Amplified plasma levels of miR-184 (in tongue carcinoma) [54], -31 [55], and -24 [29] (both in oral carcinoma) have been detected when compared with case-controlled individuals. miR-125a and -200a are two salivary miRs that have been shown to be substantially reduced in oral carcinoma patients versus healthy controls [56]. Moreover, plasma miRs were shown to be reduced after tumour resection, implying that these miRs may be released from cancerous tissues into circulation and their potential use as a marker for disease progression [54, 55]. This was seen in plasma and salivary miR-31 [57] and plasma miR-184 [45]. Further research is required in this area but positive results may lead to noninvasive diagnostic tests that will enable surgeons to determine if the margins are clear on a molecular level, which may decrease metachronous recurrence rates. Circulating miRs therefore have the potential to be powerful, noninvasive HNSCC biomarkers [15].

3. miRs in the Transition from Dysplasia to HNSCC

Despite the increasing number of studies on miR expression in HNSCC, there remain few publications that have investigated the deregulation of miRs in the transition process from dysplasia to malignancy.

In an investigation of miR precursors in oral leukoplakias (OL), Xiao et al. found an upregulation of both miR-31 and miR-31* [58]. miR-31* was negatively associated with recurrent/newly formed oral leukoplakias, and they hypothesized that miR-31* may play an important role during OL progression via the regulation of fibroblast growth factor 3 (FGF3) [58]. This was consistent with miR expression profile findings in the prospective translational study by Lajer et al. [40], who examined the global miR expression in a series of consecutive tumours or biopsies obtained from patients with OSCC and pharyngeal SCC. Of the one hundred and fourteen miRs differentially expressed between OSCC and normal epithelium, the upregulation of miR-31 and downregulation of miR-375 were found as the most significant aberrations

[40]. There is thus evidence to suggest that the upregulation of miR-31 may be an early event in the transition process from dysplasia to OSCC; however, clear elucidation of its role in tumour progression and of its predictive value still requires further investigation.

Clague et al. conducted a case-control study investigating the association between miR-related gene polymorphisms and risk of oral potentially malignant lesions (OPML) [59]. It was found that an increased risk of OPML was noted with increasing number of unfavourable genotypes, with patients with at least one variant allele of mir26a-1:rs7372209 having a significantly increased risk of OPML [59]. However, due to the retrospective nature of the study and lack of patient followup, they were unable to conclude the viability of these OPML risk alleles as potential markers for risk of progression to HNSCC [59].

In the only study to date investigating the miR expression profiles associated with progression of leukoplakia to oral carcinoma, Cervigne et al. [32] quantified miR expression changes in leukoplakia and same-site OSCC in 43 sequential progressive samples from 12 patients and four nonprogressive leukoplakias from four different patients. They succeeded in identifying an miR signature associated with progression, which was also validated using quantitative RT-PCR in an independent cohort of 52 progressive dysplasias and OSCCs and five nonprogressive dysplasias [32].

Cervigne et al. found that miR-21, miR-181b, and miR-345 were consistently increased in oral dysplasia and associated with lesion severity, with global miR expression profiles being able to distinguish progressive leukoplakia/OSCC from nonprogressive leukoplakias/normal tissues [32]. Overall, one hundred and nine miRs were highly expressed exclusively in progressive leukoplakia and invasive OSCC, with a multi-miR prognosis predictor built consisting of a set of eight miRs derived using a classical training-testing set of samples. They concluded that overexpression of miR-21, miR-181b, and miR-345 had highly significant associations with progressive leukoplakia lesions and as such could play a role in malignant transformation and may potentially be useful as an miR signature for identifying leukoplakias at risk of malignant transformation [32]. All three of these miRs were upregulated in the OSCC samples as profiled by Lajer et al. in 2011 [40]. miR-181b was also found to be more highly expressed in patients with lymph node metastases from oral cavity cancer; however, it was noted that the number of patients with lymph nodes metastasis included in the study was too low to allow for the formulation of a distinct signature, and the changes noted were moderate [40]. Nonetheless, these observations lend evidence to suggest that these miRs may have diagnostic as well as prognostic value.

4. miRs in HNSCC Surgical Margins

To date, there has only been one study investigating the role of miR in surgical margins. Santhi et al. analysed 72 miRs reported to be differentially expressed in OSCC and detected decreased expression of miR-125a, miR-184, and miR-16 and an increased expression of miR-96 in both progressive oral mucosal samples and dysplastic surgical margin samples [60]. Further studies are required to define a broader set of miR profiles within a wider range of surgical specimen samples and to correlate results with patient outcomes.

5. miR Deregulation in HNSCC Related to Specific Risk Factors

5.1. HPV-Negative HNSCC (Tobacco, Alcohol, and Areca Nut Use). Oral and laryngeal carcinomas are the most common types of HNC in smokers, who are ten times more likely to experience cancer than nonsmokers, while pharyngolaryngeal carcinomas commonly develop with alcohol intake [61, 62]. The concurrent use of tobacco and alcohol has a synergistic (greater than multiplicative joint) effect on the risk of developing HNSCC [3, 6]. The expression of miR-155 circulating in plasma/serum has been associated with a greater risk of oesophageal carcinoma in tobacco and alcohol users [63]. Through multivariate analyses, Avissar et al. [52] found miR-375 expression levels to increase with alcohol ingestion, with higher expression in pharyngeal and laryngeal tumours.

Overexpression of miR-23a has been linked to areca nut extract exposure [64]. It also targets Fanconi anaemia complementation G (FANCG), inhibiting its expression and thus promoting oncogenesis [64]. Hung et al. identified an upregulation of mir-146a associated with area nut extract exposure, which subsequently enhanced the oncogenicity of OSCC cells [65]. A recent study into miR gene polymorphisms and susceptibility to environmental factors leading to oral carcinoma found four miR gene polymorphisms which may have increased susceptibility to oral carcinoma associated with betel nut chewing and tobacco [66].

Studies have implicated cigarette smoke in miR deregulation [67, 68] with the high toxicity and mutagenicity of cigarette smoke which correlated in lung carcinomas with damage to miRs located at fragile sites of the genome [69, 70] and with deregulation of miR regulatory mechanisms such as the p53 pathway [71, 72]. A recent study using tiling low-density arrays identified widespread changes in the miR expression profile of oral fibroblasts exposed to cigarette smoke condensates, promoting a phenotype which increases oral cancer migration [73].

5.2. HPV-Positive HNSCC. There has been a recent rise in tongue and oropharyngeal carcinomas that are unrelated to the use of tobacco products, while the incidence of other HNSCCs has decreased in the United States [74–78]. The human papillomavirus (HPV) has been detected in 26.2% of dysplastic leukoplakia and other potentially malignant intraepithelial oral neoplasms [79], emerging as a major aetiological factor and creating a new and enlarging subset of HNSCC [74, 80].

Lajer et al. [14] identified a set of core miRs implicated in the known HPV pathogenesis in HPV + HNSCC, namely, miR-15a/miR-16/miR-195/miR-497 family, miR-143/miR-145, and the miR-106-363 cluster. Gao et al. [81] investigated the miR profile in HPV+ oropharyngeal SCC and found that five miRs were significantly correlated—miR-9, -223, -31, -18a,

and -155. Wald et al. [82] studied the miR expression profile in HPV16+ and HPV-HNSCC cell lines. miR-363, -33, and -497 were upregulated, while miR-155, -181a, -181b, -29a, -218, -222, -221, and -142-5p were downregulated. In a recent study, Hui et al. [83] found upregulated miR-20b, -9, and -9* associated with HPV/p16 status in oropharyngeal carcinoma. They also identified three candidate prognostic miR sets significantly associated with overall survival (miR-107, -151, and -492), disease-free survival (miR-20b, -107, -151, -182, and -361), and distant metastasis (miR-151, -152, -324-5p, -361, and -492), independent of p16 status [83].

In summary, as cited in a recent review article, Let-7, miR-125a/b, miR-200a, miR-133a/b, and miR-100 are considered tumour-suppressive miRs in HNSCC, while miR-106b-25 cluster, miR-17-92 polycistron, and miR-106a are oncogenic miRs in HNSCC [5]. Expression of miR-245, -21, and -181b is increased in leukoplakias transforming to OSCC and invasive OSCC [5]. The ratio miR-221 : miR-375 may be significant in discerning malignant HNSCC from normal tissue [5]. miR-205 has been associated with lymph node metastases [5].

It is evident that the results of most of these studies are conflicting and inconsistent, and no clear pattern has emerged [40]. Reasons for this may be due to the use of cell lines in some studies and formalin fixed paraffin embedded (FFPE) tissue samples in others, with different methods used for pathological staging and grading [40]. Samples may be from varying anatomical sites [40]. Cell lines cannot recapitulate miR profiling of solid tumours since culture conditions and clonal selection may drastically alter miR expression [40] but are used since they are inexpensive, easy to manipulate, and easily available for research purposes [84]. Moreover, the ongoing discovery of new miRs compels continued profiling in search of appropriate diagnostic and prognostic biomarkers [40].

There are large changes in miR expression compared to mRNA expression between normal cells and cancer cells thus aiding detection of differences [85]. miRs are also less prone to degradation and modification in FFPE tissue samples thus expediently supplying significant retrospective information [85]. It has been found that alterations in single miR expression have only a modest impact on individual protein expression [86, 87]. Global miR screening is therefore considered to be more useful in order to study collective changes in miR expression, since it is more likely that miRs work cooperatively *in vivo* to physiologically regulate proteins [88]. In accordance with this, Lajer et al. [14] observed that deregulation of a single miR implicates the collective action of a cluster of miRs or a whole miR family, consequently influencing multiple proteins in a complex manner.

6. miRs and Epigenetics

Epigenetic modifications at the promoter regions of genes (generally DNA methylation and histone modifications) and miR regulation at 3′-UTRs have emerged as two major regulatory mechanisms in eukaryotes, both of which can suppress gene expression [10]. It is likely that these two systems may complement each other since miRs tend to target genes with a low DNA methylation level in their promoter regions [10]. miRs are regulated by epigenetic mechanisms, similar to protein-coding genes, and the overexpression or underexpression of specific miRs in specific tumour types is a result of epigenetic aberrations in these tumour cells [10, 89]. These mechanisms include DNA methylation of miRs and aberrant expression of specific epigenetic regulators such as histone deacetylases (HDACs) or polycomb repressor complexes (PRC1 or PRC2) [10]. Since it has been found that approximately half of miR genes are associated with CpG islands, altered DNA methylation is a likely mechanism of miR regulation [16]. For example, in oral carcinoma, miR-137 is regulated by hypermethylation of the epigenetic targets—Cdk6, E2F6, NcoA2, and Lsd-1 [10].

There is also evidence that miRs also have specific epigenetic functions suggesting a fine-tuned feedback system [10, 16, 89]. This is achieved by firstly controlling the expression of important epigenetic regulators such as DNA methyltransferases, HDACs, PRC1, and PRC2—such miRs are termed epi-miRs [10]. Secondly, miRs may also have direct epigenetic functions by recruiting specific protein complexes to the promoter regions of genomic DNA [10]. Endogenous miRs are also able to activate or repress promoters directly, thus inducing or repressing specific genes directly [10]. Thus there is strong interdependence between the two gene regulatory mechanisms, and they are not entirely separable in their cooperation to establish the gene expression profile in specific cells [10]. Disruptions to this intricate network lead to various diseases including cancer [10].

7. Salivary Diagnostics

Saliva is an attractive medium for HNSCC biomarker detection due to the noninvasive nature of collection, its status as a proximal biofluid in the context of HNSCC, and the multitude of biomarkers that may be used to detect neoplastic change [90, 91]. Although the concept of salivary diagnostics in cancer is appealing, there has been difficulty in realising this due to the complexity surrounding HNSCCs as well as constituents in saliva [92–94].

Previous research into saliva as a source for biomarkers focused on proteomics [92], which has proved to be difficult due to protein polymorphism in saliva, protein instability, and degradation in stored samples [92] and due to saliva substrate concentrations being about a thousandfold less than those in blood [95].

With the discovery of miRs and their importance in the regulation of cellular processes, there has been an increasing interest in the potential utility of salivary miRs in HNSCC diagnostics and prognosis. In addition, it was reported that miR concentrations were higher in saliva compared to other bodily fluids [96]. Weber et al. compared miR expression levels in 12 body fluids, including traditionally used fluids such as plasma, and concluded that saliva had the highest concentration of miRs [96]. However, there remains a lack of studies investigating salivary miR levels in HNSCC. Liu et al. first reported the heightened miR-31 levels in saliva and plasma of OSCC patients with a subsequent decrease in

miR-31 levels after surgery [55]. It should be noted however that the study did not conduct a sensitivity analysis due to a small sample size ($n = 43$). A subsequent study conducted by the same group compared salivary miR-31 levels of OSCC against oral verrucous leukoplakia (OVL) patients, with findings of significant differences in OSCC with OVL and healthy subjects [57]. This is significant as OVL has been reported to have a relatively higher risk of turning neoplastic [97], and this further hints at a potential utility of salivary miR diagnostics in discriminating between neoplastic and benign lesions. Further investigations should be conducted to better define the discriminatory power of salivary miR levels. In addition, the study also found greater miR-31 levels in the saliva of OSCC patients compared to those in the plasma [57]. The study concluded that salivary miR-31 as a cancer biomarker had greater sensitivity than that using plasma [57]. Langevin et al. found that there were higher miR-137 methylation levels in salivary mouth rinses of HNSCC patients [98], with a follow-up study concluding that patients with higher miR-137 methylation levels were associated with lower survival rates [99]. Given the current low literature base, more research is required to better elucidate the role of salivary miRs in HNSCC diagnostics and prognosis.

8. Methodologies Used in Salivary miR Assays

Salivary diagnostics is unique in that the miR numbers can be up to a thousandfold less than those found in fresh tumor samples [100], as such there is an increased importance placed on proper sample collection and assessment methods. Normalisation for quantitative real-time polymerase chain reaction (qRT-PCR) assays has emerged as a significant source of differences between research groups. Normalisation for variations in miR values and cDNA synthesis is essential [101] between the cancer and healthy groups, especially with salivary miR [100] in order to allow comparable findings across studies. Two widely used normalisers in the literature for HNSCC miR analysis are endogenous small-nucleolar RNAs (snRNAs) such as RNU44, RNU48, RNU43, and RNU6B [102] as well as endogenous miRs such as Let-7a, miR-16, and miR-191 [103]. snRNA expression has been shown by Gee et al. to be variable in HNSCC patients, where the use of snRNA as normalisers acted to bias miR expression values [102]. There is also accumulating evidence of snRNAs playing a role in cancer [104]. Although the use of endogenous miRs in normalisation has been qualified in breast [105] and colorectal [106] cancers, there are no studies in the literature investigating the use of these miRs in HNSCC. This is important, as there is evidence that there are measurable changes in Let-7a [45, 47, 107, 108], miR-16 [45, 47], and miR-191 [50] levels in the context of HNSCC from the literature.

In order to prevent problems with tissue context, some authors have described using a mean expression ratio of miRs expressed as a normalizer [109]. This approach has been qualified [110] and is well accepted [100]. Another approach is the use of synthetic control miRs that are spiked in before analysing to normalise the sample [111]. This approach has the advantage of having a quantifiable reference point that will not change [111]. However, some issues may arise with ensuring a homogenous quantity between experiments [100]. In view of these, investigators will be well served in choosing the right normaliser in the context of tissue types in conducting qRT-PCR to ensure that comparable results across studies are obtained.

There are two main forms of salivary samples used in the literature—whole saliva and the cell-free, supernatant phase that is obtained from progressive centrifugation. There has been debate over which is the more informative sample type since Park et al. first reported a different miR profile in the two samples, with the conclusion that the supernatant phase of saliva was the more informative one [56]. However, Patel et al. disagreed, stating that there was a higher resolution in the miR profile from whole saliva [90]. Spielman et al. were in agreement with Park et al. after using next generation sequencing (NGS) techniques to profile the RNA transcriptome in both phases of saliva [112]. This profiling technique is generally accepted to be the gold standard due to its accuracy and the ability to discern novel miRs [100]. Indeed, this debate in salivary diagnostics is not new, with previous research on salivary mRNA diagnostics raising similar contradictions [113–115]. The evidence appears to show that the supernatant phase of saliva contains more relevant miRs. In addition, reports of miR-containing, extracellular exosomes that resist degradation have bolstered the argument for using the supernatant phase in assay studies.

9. Exosomal miRs in Saliva

Cell-free, endogenous salivary miR has been found to degrade at a much slower rate compared to nonsalivary miR when exposed to salivary ribonucleases [56, 116]. Moreover, Patel et al. found that Let-7b levels remained fairly constant when spiked with a RNA stabilizing agent and stored in room temperature for over 3 days [90]. There are two main hypotheses in the literature explaining this phenomenon. The first is that the miRs are packaged in exosomes, enabling the miRs contained within to be protected against degradation by salivary ribonucleases [117].

Exosomes are cell-secreted vesicles of 30–100 nm derived from the fusion of multivesicular bodies to plasma membranes [118] and are contained in the supernatant of saliva [119]. Exosomes have been thought to allow intercellular communication [120] through horizontal transfer between cells in different anatomic sites [121], as well to protect miRs from thermal, pH, freeze-thaw cycles, and extracellular ribonuclease [122] and is thought by some authors as the major source of miRs in saliva and serum [119]. Arroyo et al. contest this [123] although it should be noted that the study investigated plasma miR. There are no studies in the literature investigating the concentration of miR outside of exosomes in the supernatant phase of saliva.

The second hypothesis is that these miRs form a protein complex with Argonaute 2, increasing its stability in plasma and serum [123]. Although one group has found that up to 90% of miRs in plasma and serum are found in these complexes instead of in exosomes [123], there are no studies in the literature that have investigated this hypothesis in saliva.

10. Novel Therapeutic Targets: The Small and Powerful

In addition to having considerable diagnostic and prognostic value, miRs are potential therapeutic targets or therapeutic agents depending on the type of mRNA(s) they affect [14]. miRs provide an unparalleled opportunity to target multiple molecules, usually in the context of a network, making them efficient in regulating distinct biological cell processes [16]. Synthetically designed miR mimics (agomiRs) [124], miR inhibitors (such as anti-miR oligonucleotides; AMOs) [41], miR antagonists ("antagomiRs") [125, 126], and "miR sponges" [127] are some innovative methods of modulating oncogenic or tumour-suppressive pathways for therapeutic purposes.

Strategies to target miRs in cancer may be direct, involving the use of oligonucleotides or virus-based constructs to either inhibit oncomir expression or to upregulate tumour suppressor miR expression, or indirect, where drugs are used to modulate miR expression by targeting their transcription and processing [16]. However, a number of challenges prevail in the development of a specific and efficient drug delivery system for miR-based drugs [15, 16]. Therapeutic RNA must exit the circulatory system, transit the cell membrane, and avoid endosomal vesicles to gain entry into the cytoplasm and access the target site [16]. Furthermore, nonconjugated therapeutic RNA (7–20 kDa) is likely to be cleared by phagocytic immune cells or by the kidneys, which filter out molecules less than 50 kDa [16]. While there is limited information on miR-based therapeutic targets in HNSCC, the following is a brief description of known miR therapeutic agents and targets.

Mature miR levels may be reduced by exogenously delivering synthetic double-stranded hairpin by complexing with lipids or delivery proteins: introduction of miR-34a induced apoptosis of experimental lung metastasis of murine melanoma and suppressed cellular proliferation in two colon cancer cell lines and may also be effective in HNSCC cells [128, 129]. Unmodified dsRNAs are likely to be degraded by nucleases *in vivo*, limiting the use of this class of compound to privileged local environments where local administration is possible (e.g., intranasal delivery in mouse lung cancer model) [85]. For stable miR reintroduction, the expression can be enforced by a viral vector with Pol III promoters, which have the advantage of providing high expression of miRs from well-defined transcription start and termination sites [129–131]; however, they have no cell specificity [16]. In contrast, RNA Pol II promoters can express pri-miRs, allowing for tissue-specificity or induced ectopic miR expression [132]. The latter two methods may be effective in the reintroduction of downregulated miRs in HNSCC (e.g., miR-375 [40]).

However, there are many hazards of reintroducing an miR with a viral system [16]. The site of integration of the delivered material to host DNA is unpredictable, with the associated risks of insertional mutagenesis and activation of protooncogenes [16]. The use of retroviral vectors (which, along with lentiviral vectors, integrate their DNA into the host genome) is limited to actively dividing cells, while adenoviral vectors (which remain unintegrated into the host genome, replicating as an autonomous unit) tend to induce a strong immunological response [16, 133].

AntagomiRs are a class of chemically engineered oligonucleotides able to silence endogenous miRs [16, 126]. AntagomiRs of upregulated miRs in HNSCC would revert to the effects of upregulation; for example, delivery of antagomir-155 into KB cells overexpressing miR-155 in nude mice decreased cell viability and increased apoptosis [125]. The interfering nanoparticle (iNOP) is a novel therapeutic agent which may be complexed with antagomiRs and delivered intravenously, enhancing the delivery of the antagomiR [134]. The effects of iNOP-stabilised anti-miR-122, which silenced miR-122 in mice, were found to be long lasting and did not induce an immune response [134].

miR loss of function may be achieved by AMOs (chemically modified anti-miR oligonucleotides), which have high specificity and binding affinity to RNA but lack an effective delivery mechanism into target tissues [41, 128]. Inhibition of mir-21 with AMO in tongue SCC cell lines has been shown to induce apoptosis and reduce survival and anchorage-independent growth [41]. Furthermore, repeated injection of miR-21 AMO suppressed tumour formation in nude mice reducing cell proliferation and inducing apoptosis [41].

Oncomirs in HNSCC may be inhibited using miR sponges or miR-masks. miR sponges are miR inhibitory transgenes expressing an mRNA containing multiple tandem binding sites for an endogenous miR (with the potential to block entire miR families), thus able to stably interact with the corresponding miR and prevent the association with its endogenous targets [16, 127]. In contrast, miR-masking antisense oligonucleotides technology consists of fully complementary antisense oligonucleotides to complementary miR binding sites in the 3′-UTR of a specific target mRNA, thus disrupting the interaction between specific miR-mRNA pairs [16, 131]. While unwanted effects or off-target effects are greatly reduced in this method, this might be a disadvantage in cancer therapy where targeting multiple pathways might be desirable [16].

In addition to targeted therapies and chemotherapies, miRs could also alter sensitivity to radiotherapy [132], suggesting that the activation or suppression of implicated miRs would enhance the outcome of radiotherapy. Furthermore epigenetic drugs (e.g., DNA demethylating agents and histone deacetylase inhibitors) are capable of reversing irregular methylation or acetylation [16]. The action of such drugs could restore tumour-suppressive miR expression and revert a tumoral phenotype [16].

The majority of the aforementioned methods are in the experimental phase, but development of animal models to study cancer-associated miRs and improvement of miR/anti-miR delivery efficiency *in vivo* are fundamental to translating these research advances to medical practice [16]. Furthermore, most of these strategies target a single miR or a family of miRs [16]. Since multiple miRs coordinate in cancer pathogenesis with multiple miR-transcriptome interactions, future strategies should aim to reprogramme aberrant miR networks in cancer [16]. This may be achieved by targeting components of miR biogenesis machinery or elements of regulatory networks (such as the epigenetic programme) [16, 133]. Furthermore, it has been shown that miRs are actively reexpressed after treatment with these drugs, contributing to their

therapeutic benefits [16]. Nevertheless, the full potential of these drugs remains to be verified.

11. Conclusion

Current research has identified deregulated miRs in HNSCC oral premalignant lesions; however, a wide range of miRs have been implicated, regulated by cellular and epigenetic factors. miR signatures with diagnostic accuracy in tumour tissue samples, premalignant lesion samples, and saliva samples are yet to be validated in large clinical trials. This would lead to the development of novel therapeutic targets and agents with the overarching aim of providing customised patient-centred management leading to improved prognosis for HNSCC patients.

Conflict of Interests

Camile S. Farah is the Guest Lead Editor of the special issue on oral cancer and oral potentially malignant disorders for the International Journal of Dentistry. This paper has undergone independent external peer review, and has been handled by one of the other guest editors.

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Research Article

Three-Dimensional Reconstruction of Oral Tongue Squamous Cell Carcinoma at Invasion Front

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We conducted three-dimensional (3D) reconstruction of oral tongue squamous cell carcinoma (OTSCC) using serial histological sections to visualize the architecture of invasive tumors. Fourteen OTSCC cases were collected from archival paraffin-embedded specimens. Based on a pathodiagnostic survey of whole cancer lesions, a core tissue specimen (3 mm in diameter) was dissected out from the deep invasion front using a paraffin tissue microarray. Serial sections (4 μm thick) were double immunostained with pan-cytokeratin and Ki67 antibodies and digitized images were acquired using virtual microscopy. For 3D reconstruction, image registration and RGB color segmentation were automated using ImageJ software to avoid operator-dependent subjective errors. Based on the 3D tumor architecture, we classified the mode of invasion into four types: pushing and bulky architecture; trabecular architecture; diffuse spreading; and special forms. Direct visualization and quantitative assessment of the parenchymal-stromal border provide a new dimension in our understanding of OTSCC architecture. These 3D morphometric analyses also ascertained that cell invasion (individually and collectively) occurs at the deep invasive front of the OTSCC. These results demonstrate the advantages of histology-based 3D reconstruction for evaluating tumor architecture and its potential for a wide range of applications.

1. Introduction

Oral tongue squamous cell carcinoma (OTSCC) is the most prevalent head and neck cancer, and the presence of occult neck metastases is the main predictor of outcome in patients with early-stage OTSCC [1–4]. In the literature, it has been documented that the depth of infiltration of the primary tumor correlates significantly with the rate of regional nodal metastases [5–7], but the specific cut-off value for the depth of infiltration distinguishing high-risk and low-risk patients remains controversial [8, 9]. Most recently, Ganly et al. [10] indicated that patients with pathologic T1-T2/N0 OTSCC had a greater than expected rate of neck failure and that failure occurred predominantly in patients with primary tumors greater than 4 mm in thickness.

It is well known that the histological features of OTSCC differ widely between different regions within the same tumor, and much effort has been given to explore specific invasion patterns that may have prognostic value. A consensus is developing that different patterns of tumor invasion are determined by the interaction between cancer cells and the circumscribing stromal environment [11, 12] and that the most useful prognostic information can be deduced from the deepest invasive front of the tumor, where the most aggressive cells are presumably found [13–15]. In the literature, the deep invasion patterns of OTSCC are usually classified into three

Table 1: Clinicopathological features of 14 patients with OTSCC.

OTSCC cases	Age (yrs)	Sex	p-T	Growth pattern[(1)]	Depth of invasion[(2)] (mm)	Occult metastasis
A	51	M	T1	SS	1.9	−
B	59	M	T1	SS	2.4	+
C	82	F	T2	EN	2.5	+
D	52	M	T2	SS	2.6	−
E	53	M	T1	EX	2.8	−
F	52	M	T2	EX	2.9	+
G	60	M	T1	SS	3.3	−
H	78	M	T2	EN	3.5	+
I	55	F	T1	EX	3.6	−
J	57	M	T1	SS	4.6	+
K	40	F	T2	EN	7.0	+
L	76	F	T2	EN	7.6	+
M	69	F	T1	EN	10.6	+
N	33	M	T2	EN	N.A.	−

[(1)]Macroscopic growth patterns of OTSCC were classified into superficial spreading (SS), exophytic (EX), and endophytic (EN) types.

[(2)]The depth of invasion was measured from the level of the adjacent normal mucosal surface to the deepest portion of tumor invasion. In case N, the measurement was not applicable (N.A.).

or four categories, such as (i) pushing types with well-delineated infiltrating borders; (ii) diffuse types with infiltrating solid cords, bands, and/or strands; and (iii) destructive types with widely spread cellular dissociation in small groups and/or single cells [8, 16–18]. With respect to the relationship between the invasion pattern and clinical outcome, many studies conclude that the prognosis of patients whose OTSCC exhibits a pushing-border invasion pattern is better than for those with diffuse and destructive invasion types [8, 14–18]. Nevertheless, the propensity of an OTSCC to metastasize subclinically remains hard to predict solely through microscopic examination [19, 20].

An important consideration is that histopathological features of tumor malignancy are usually diagnosed from two-dimensional (2D) microscopic findings, whereas tumor progression actually occurs within a three dimensional (3D) microenvironment. Recent developments in computing power and software sophistication have facilitated the 3D reconstruction of anatomical and pathological structures [21–24]. We previously described the development of technology to reconstruct the tumor-host environment using serial histological sections of archival paraffin-embedded human cancer specimens [25]. In the current paper, we use double immunostaining with pan-cytokeratin and Ki67 antibodies to correlate microscopic 2D and 3D findings with respect to heterogeneous tumor architecture in T1/T2 OTSCC at the deepest invasion front. The main objective of the present study was to visualize directly the tissue architecture at the tumor-host interface and to assess quantitatively the mode of invasion for single cells and cell clumps within the 3D microenvironment.

2. Materials and Methods

2.1. Patients and Tongue Cancer Cases. For the present study, we included 14 patients with early-stage (cT1-T2/N0) OTSCC who were treated at Nippon Dental University and Saitama Cancer Institute between 2000 and 2009. Patient records were retrieved from the archival OTSCC records based on the following criteria: (a) patients had radical surgery as their first line of management (i.e., without preoperative chemotherapy and radiotherapy); (b) paraffin-embedded tissue specimens of the primary tumor were available; and (c) clinical follow-up data for a minimum of 24 months or until death were recorded. The patient group included nine men and five women with a mean age of 58 years (range: 33–82 years) at the time of diagnosis. Although all patients were N0 at presentation, eight had occult neck lesions discovered during the follow-up without failure at the primary site. Based on microscopic images of whole tumor lesions (see Supplementary Material Plate S1 available online at http://dx.doi.org/10.1155/2013/482765), tumor growth patterns were classified into three types, that is, superficial spreading, exophytic, and endophytic. The clinical and pathological features of these OTSCC cases are shown in Table 1. The use of archival human tissues in this study was in accordance with the provisions of the Declaration of Helsinki for research involving human tissue and was approved by the Institutional Review Boards of Nippon Dental University and Saitama Cancer Institute.

2.2. Preparation of Histological Sections. We first reexamined primary tumor lesions from the 14 patients by preparing both HE-stained and cytokeratin-immunostained sections from the archival formalin-fixed, paraffin-embedded tissue blocks. The depth of invasion was measured from the level of the nearest adjacent normal mucosa to the extent of the deepest tumor invasion into the tongue musculature. Taking into account the observed locoregional heterogeneous histological features, we collected core specimens of 3 mm in diameter from multiple deep invasion sites of the whole tumor lesion using tissue-array apparatus (Model KIM-I, Azumaya, Tokyo, Japan; Figure 1(b)). Three-dimensional reconstruction and

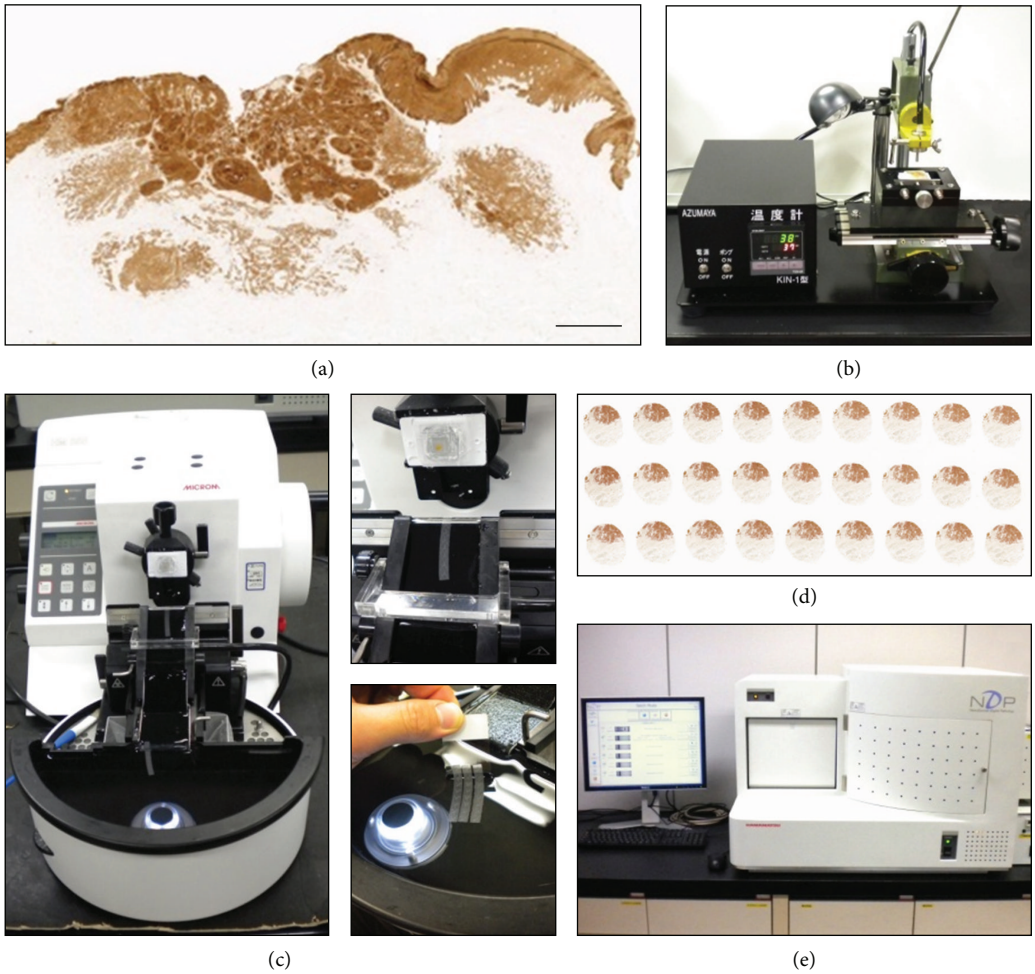

Figure 1: (a) Original pan-cytokeratin-stained image of an OTSCC lesion. Bar = 1 mm. (b) Tissue-array apparatus used to collect a core biopsy of 3 mm in diameter from the deepest invasion front (see Plate S1). (c) Preparation of serial sections (4 μm thick) from the core specimen using a rotary microtome. The serial sections were conveyed *via* continuous laminar water flow directly from the knife edge to the heated water bath. (d) Three rows of 9 sections were mounted on each glass slide for immunostaining with Ki67 and CK cocktail. (e) All histological images were digitized using virtual microscopy.

morphometric data are presented for a single representative site for each SCC case (Supplementary Material, Plate S1). The selected tissue core specimen was reembedded in paraffin and consecutive histological sections of 4 μm thickness were prepared using an electronic motorized rotary microtome (Microm HM 355S, MICROM International GmbH, Walldorf, Germany; Figure 1(c)), which has been used successfully by others to prepare serial sections [22]. One crucial technical point is the use of a tissue core specimen of 3 mm in diameter, which enabled us to consistently obtain from the region of interest 80–120 serial sections of equal thickness and with minimal distortion (Figure 1(c)). These serial sections were mounted on glass slides, usually in three rows of 8-9 sections (Figure 1(d)). Because it minimizes the number of glass slides used for immunostaining and, as described later, facilitates automated image registration, this regimented arrangement of circular serial sections on a glass slide aided the efficient execution of our histology-based 3D reconstructions, which would otherwise be rather time and labor intensive.

2.3. Immunohistochemistry. We first established the immunoreactivity profiles of OTSCC lesions using a range of anticytokeratin antibodies, because cancer cells are usually heterogeneous with respect to their cytokeratin expression. From this profiling, we selected a cocktail of three antibodies (AE1/AE3, 34βE12, and MNF116; hereafter called CK) for our 3D reconstruction, and this CK cocktail highlighted infiltrating cancer cells and clumps at the invasion front as described in the literature [20]. Before immunostaining the serial sections, slides were deparaffinized in xylene for 3 × 5 min, rehydrated through graded ethanol to distilled water, incubated for 20 min with 3% hydrogen peroxidase in PBS to inhibit endogenous peroxidase activity, and then heated in TE buffer (0.01 M Tris-HCl plus 1 mM EDTA; pH9.0) for 60 min at 98°C for antigen retrieval. After cooling to room temperature (~60 min), the sections were incubated in 5% skimmed milk in PBS for 20 min at room temperature to block nonspecific protein binding. For double immunostaining, the sections were first incubated overnight at 4°C with Ki67 primary

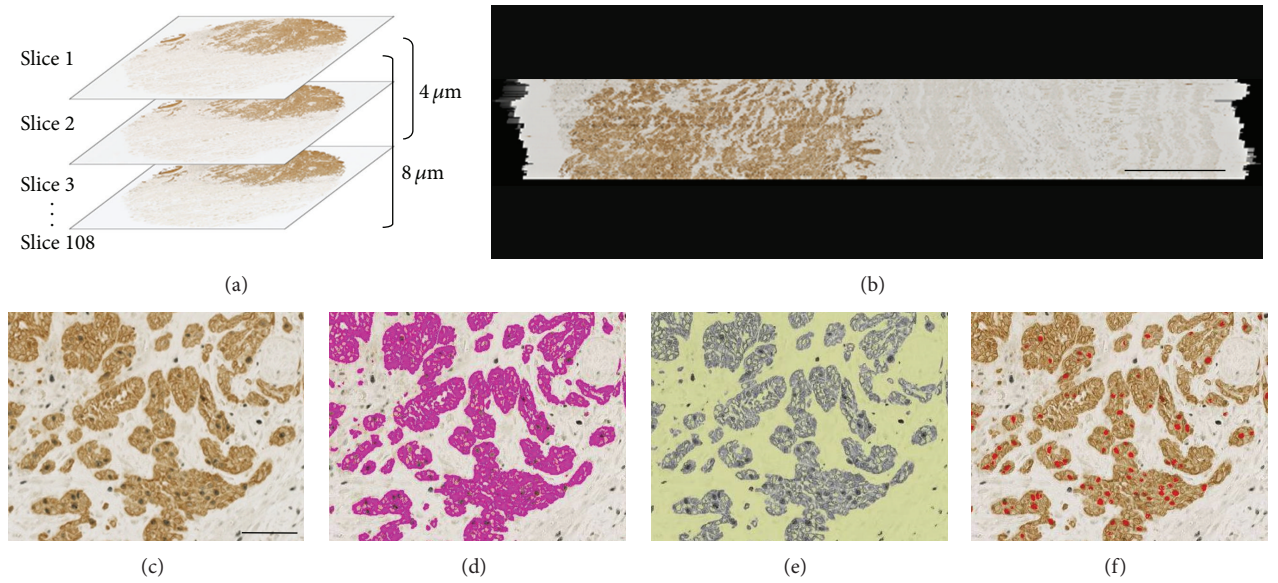

Figure 2: (a) Image registration of consecutive histological images in the z-axis. (b) Sagittal cross-cut view through the center of an image stack comprising 108 serial sections, showing successful superimposition without marked irregularity or distortion in interior tissue geometry. Bar = 0.5 mm. ((c)–(e)) Segmentation of cancer cells and Ki67-positive nuclei: (c) original double-immunostained image with DAB and Vector SG as chromogens; (d) segmentation of CK-positive cancer cell cytoplasm, shown as purple pseudocolor (note that Ki67-negative nuclei remained open within the segmented cytoplasm); and (e) stromal region (yellow) after subtraction of the tumor parenchyma (see text for details); (f) Ki67-positive nuclei (red) attained by ImageJ SG color segmentation. Bar = 50 μm.

antibody (MIB-1, 1 : 100; DAKO, Tokyo, Japan) and then developed using Vectastain Elite ABC kits (mouse; Vector Laboratories Inc., Burlingame, CA, USA). Vector SG (SK-4700, Blue/Gray; Vector Laboratories Inc.) was used as the substrate for the peroxidase-mediated reaction. After Ki67 immunostaining, the sections were reheated in TE buffer in a microwave oven for 5 min at 90°C for antigen retrieval. For detection of tumor parenchyma using the CK cocktail, the same sections were processed using a labeled streptavidin-biotin (LSAB) method according to the Ventana DAB Universal Kit and an automated staining apparatus (NexES IHC, Ventana Medical Systems, Oro Valley, AZ, USA). Slides were mounted with EntellantNew (MERCK, Darmstad, Germany).

2.4. Histological Image Digitization. Red-green-blue (RGB) color images of consecutive histological sections were acquired using a 20x objective with a virtual microscope (NanoZoomer HT, Hamamatsu Photonics, Hamamatsu, Japan) (Figure 1(e)). To ensure reproducible image acquisition, color balance correction was conducted using a blank reference slide standard according to the manufacturer's procedure. The manufacturer also provided viewing software for which the scanning mode can be freely adjusted to automatically capture individual circular histological areas on a glass slide according to their numerical order. After storage of an entire series of histological digitized images (resolution of 0.92 μm per pixel, 5120 × 4096 pixels, 60 MB, and 32-bit RGB TIFF format), the entire circular tumor area was transposed onto a desktop computer (see below) using frame grabber software (TRI-SRF2, Ratoc System Engineering Ltd, Tokyo, Japan).

2.5. Image Registration, Segmentation of Tissue Components, and Geometry Reconstruction. Image registration (rough and fine alignment of consecutive histological images) and segmentation (identification of the boundaries of target structures in images) were conducted using open-source ImageJ software that is recognized as providing practical solutions for managing memory and automated approaches in 3D registration and visualization [26]. Specifically, we used ImageJ plugins (StackReg, TurboReg, and Color Deconvolution) for computation of image registration and RGB color segmentation. This open-source software has the great advantage of providing color-segmentation channels specific for the DAB and Vector SG used in our double-immunostained sections.

In this study, superimposition of 80–108 consecutively captured images (Figure 2(a)) was accomplished successfully in an automated, algorithm-driven manner. By browsing through an image stack, it was verified that the tumor architecture was smoothly continuous between adjacent slices with no marked irregularity or distortion in interior tumor geometry and surface contour delineation (Figure 2(b)). One crucial condition for accomplishing the automated image registration is the preparation of regularly-oriented good-quality serial sections, since computation for alignment of serial sections depends on the performance of an approximate initial registration to correct for shifts and rotations of sections on the glass slides. It is also notable that we used no algorithmic image transformation, such as stretching or

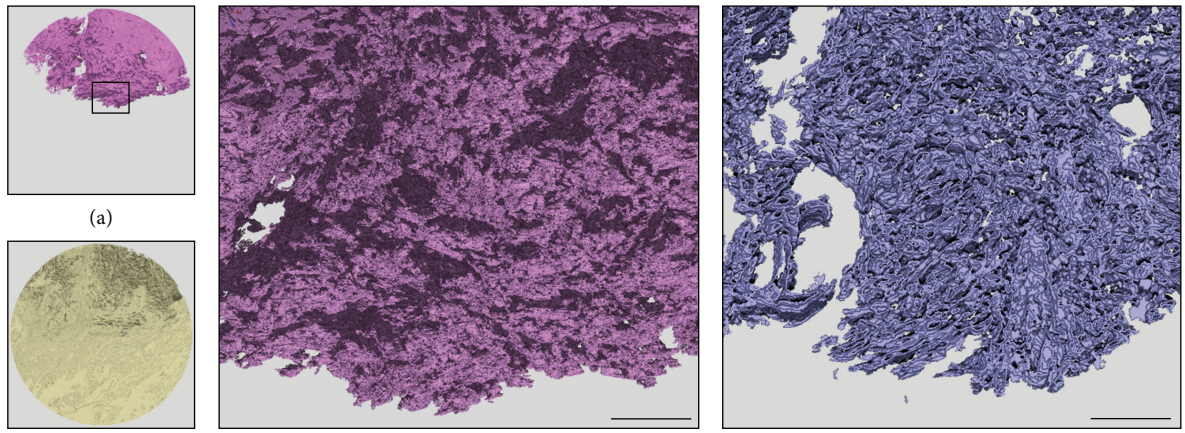

Figure 3: (a, b) 3D view of the segmented tumor parenchymal (purple) and stromal (yellow) regions, respectively. (c) Enlargement of the CK-positive tumor architecture in the rectangle shown in (a), showing in detail the interconnected tumor texture and channels or holes corresponding to penetrating stromal strands. (d) Labyrinthine architecture of the tumor parenchymal-stromal border, which was virtually displayed in a plane of one voxel in width. Note that this 3D image was constructed using 30 serial sections to improve the visibility of the tortuous architecture. Bar = 0.5 mm.

shrinking images in the x-y dimension to adjust for small misalignments between sections; in our experience of image registration, the introduction of the ImageJ affine image transformer actually results in deterioration of the quality of the image stack rather than improvement.

Image segmentation of tumor architecture (Figures 2(c)–2(e)) was performed using ImageJ DAB-color segmentation based on CK-positive immunohistochemical features of SCC cells. Following the segmentation of the tumor parenchyma volume, the RGB color-based volume data were inverted in a form of binary code. Thereafter, the stroma volume was calculated by subtracting the parenchyma volume from the whole tissue volume. According to the same computation logics, all Ki67-positive nuclei were labeled according to the ImageJ SG-color segmentation procedure (Figure 2(f)) and then segmented into two fractions: nuclei embedded within the CK-positive cancer cytoplasm and nuclei within the whole stromal volume. During this segmentation of nuclei, we adopted the criterion that all objects assigned as nuclei must be connected between at least two consecutive images. Thus, any binary signals in a single image plane with no connection to any corresponding objects in adjacent images were deleted as noise. The results of segmentation for each microscopic field were validated by overlaying the segmented elements on the original immunostained images on a computer screen.

Finally, a serial stack of images was used to reconstruct the 3D configuration of the specimen. Z-depth was adjusted to the thickness of each slice (4 μm) to give an accurate 3D representation of tissue volume. Three-dimensional volume rendering of the architecture of the whole tumor and of segmented components was performed using VG Studio Max software (Volume Graphics Ltd, Heidelberg, Germany), which improves the quality of 3D visualization by adjusting the opacity and color of target components in the reconstructed space.

2.6. Three-Dimensional Morphometry of Tumor Architecture and Proliferation Activity. Based on the 3D volumetric information obtained, quantitative assessment was conducted using RATOC SRF2 software. The parameters of interest were the volumes of the tumor parenchyma and stroma (in mm^3); the area of the segmented parenchymal-stromal border (in mm^2); and the numbers of Ki67-positive nuclei and infiltrating cancer cells or clumps. To measure cancer infiltration, we labeled and counted only cancer cells or clumps that had detached from each other and were completely surrounded by stroma. Any tumor components in contact with the outer surfaces of an image stack (i.e., both top and bottom image planes and lateral circularly-cut margins) were deleted from the pool of the putative infiltrating cancer cells or clumps. We also conducted a computational test with voxel-based dilation/shrinkage functions of the labeled components to validate the continuity of tumor architecture or detachment of cancer cells from neighboring cancer foci in the 3D space.

2.7. Computer Hardware. The computer system used to run the 3D reconstruction and visualization consisted of an Intel Xeon computer with a 3.0 GHz processor, 32 GB RAM, Windows XP professional 64-bit edition, a graphics video card NVIDIA Quadro FX 5600 with 1.5 GB RAM, a 24-inch dual monitor system, and 500 GB internal disk drive. This computer was equipped with all necessary software as described above.

3. Results

3.1. Three-Dimensional Visualization of Tumor Parenchymal-Stromal Border at the Invasion Front. Figure 3 shows an example of the segregation of the CK-positive tumor parenchyma (Figure 3(a)) and the adjacent stromal space (Figure 3(b)). In this OTSCC case (case C in Table 2), the segmented tumor

Table 2: Results of 3D morphometry of 14 OTSCC cases.

OTSCC cases	Invasion mode[1]	Tissue volume[2]		Border area (S)	S/V_p[3]	Ki67(+) nucleiin cancer cells[4]		Discohesivecancer foci[5]	
		Total mm^3	Parenchyma (V_p) mm^3 (%)	mm^2	mm^{-1}	Number	$\times 10^4/mm^3$	Number	% volume
A	DS	2.88	0.19 (6.6)	17.2	90.5	20,805	[10.9]	65	(2.83)
B	PB	2.86	0.96 (33.6)	48.3	50.3	221,803	[23.1]	2	(0.0010)
C	PB	2.95	0.56 (21.4)	61.4	97.5	61,896	[9.8]	30	(0.060)
D	PB	2.91	0.37 (12.7)	30.9	83.5	47,913	[12.9]	53	(0.0089)
E	TSC	2.74	0.47 (17.2)	18.3	38.9	23,702	[5.0]	19	(0.44)
F	PB	2.58	0.43 (16.7)	31.3	72.8	108,234	[25.1]	116	(0.26)
G	TSC	2.94	0.20 (6.8)	27.2	136.0	29,009	[14.5]	76	(0.59)
H	PB	2.19	0.92 (42.0)	81.8	88.9	88,303	[9.6]	19	(0.024)
I	TSC	2.91	0.49 (16.8)	24.4	49.8	80,410	[16.4]	7	(0.088)
J	TSC	2.59	0.11 (4.2)	11.2	101.8	18,435	[16.8]	36	(0.33)
K	SF	2.39	0.031 (1.3)	3.8	122.6	4,193	[13.5]	117	(27.1)
L	DS	2.73	0.11 (4.0)	18.4	167.3	35,156	[32.0]	232	(3.76)
M	DS	2.74	0.19 (6.9)	28.8	151.6	45,217	[23.8]	159	(8.34)
N	SF	2.91	0.032 (1.1)	27.2	850.0	9,751	[30.5]	35	(1.69)

[1]The abbreviations used are PB, pushing and bulky architecture; TSC, trabecular architecture with strands and cords; DS, diffuse spreading; and SF, special forms.

[2]The volume data correspond to the reconstructed total tissue volume and the segmented tumor parenchymal volume (V_p). The number in parentheses indicates the volume ratio of V_p to the total tissue.

[3]S/V_p indicates the ratio between tumor parenchyma-stroma border area (S) and tumor parenchyma volume (V_p).

[4]The number in brackets indicates the number of Ki67-positive nuclei per tumor parenchyma volume (V_p).

[5]The number in parentheses indicates the volume ratio of discohesive cancer foci to tumor parenchymal volume (V_p).

mass appears bulky at low magnification, but a magnified 3D visualization reveals that the parenchymal-stromal border has a rough surface texture where small tumor cords and strands are connected to each other with narrow stromal penetration (Figure 3(c)). When the segmented parenchymal-stromal border was delineated in a virtual space as a plane of one voxel in width, the labyrinthine structure extending into the massive intratumor space can be fully appreciated (Figure 3(d)). As shown in Table 2, based on 3D morphometry, this bulky tumor architecture and its intricate tumor-host border correspond numerically to a parenchymal volume of 2.95 mm^3, a border area of 61.4 mm^2, and a surface area per volume of 97.5 mm^{-1}.

3.2. Three-Dimensional Tumor Architecture at the Invasion Front. Figure 4 compares microscopic (2D) and reconstructed (3D) images of tumor architecture and invasion mode in four cases of OTSCC; the remaining data from the other OTSCC cases are presented in Supplementary Material Plates S2 and S3. Using the CK cocktail to label cancer cells, 2D views reveal heterogeneity in the patterns of cancer cell invasion both between OTSCC cases and within individual cases. Among the infiltrating cancer cells observed on 2D images, the 3D segmentation protocol enabled the visualization of discrete cancer cells and clumps that were detached from each other and completely encased in the stroma. We herein use the term "discohesive cancer foci" to designate a pool of infiltrating cancer cells segmented in 3D space. We also addressed locoregional differences in mitotic activity as indicated by the density of Ki67-positive nuclei in the parenchymal and stromal segments.Based on the 3D features of tumor architecture, we discerned four types: (1) pushing and bulky architecture with short finger-like projections; (2) trabecular architecture with strands and cords; (3) diffuse spreading; and (4) special forms.

(1) *Pushing and bulky architecture with short finger-like projections*: this type of SCC case was typified by one or a few solid tumor masses with delineated pushing infiltration at the advancing front. CK-immunostaining disclosed a fine network of tumor strands and cords as well as massive tumor growth on the 2D image. However, when viewed in 3D, we discovered that the majority of tumor strands and cords were topologically connected, giving rise to a massive tumor volume with narrow stromal penetration, as depicted in Figure 3. This 3D segmentation also verified the presence and localized the distribution of discohesive cancer foci along the tumor-host border. Two common trends were noted in the mitotic activities of the parenchyma and stroma: (a) the tumor periphery in contact with the region of stromal penetration usually showed higher mitotic activity than the compact core of the tumor mass and (b) dense Ki67 nuclear staining in the narrow stromal margins adjacent to the pushing tumor mass, most of which was attributable to infiltrating leukocytes and proliferating endothelial cells (data not included).

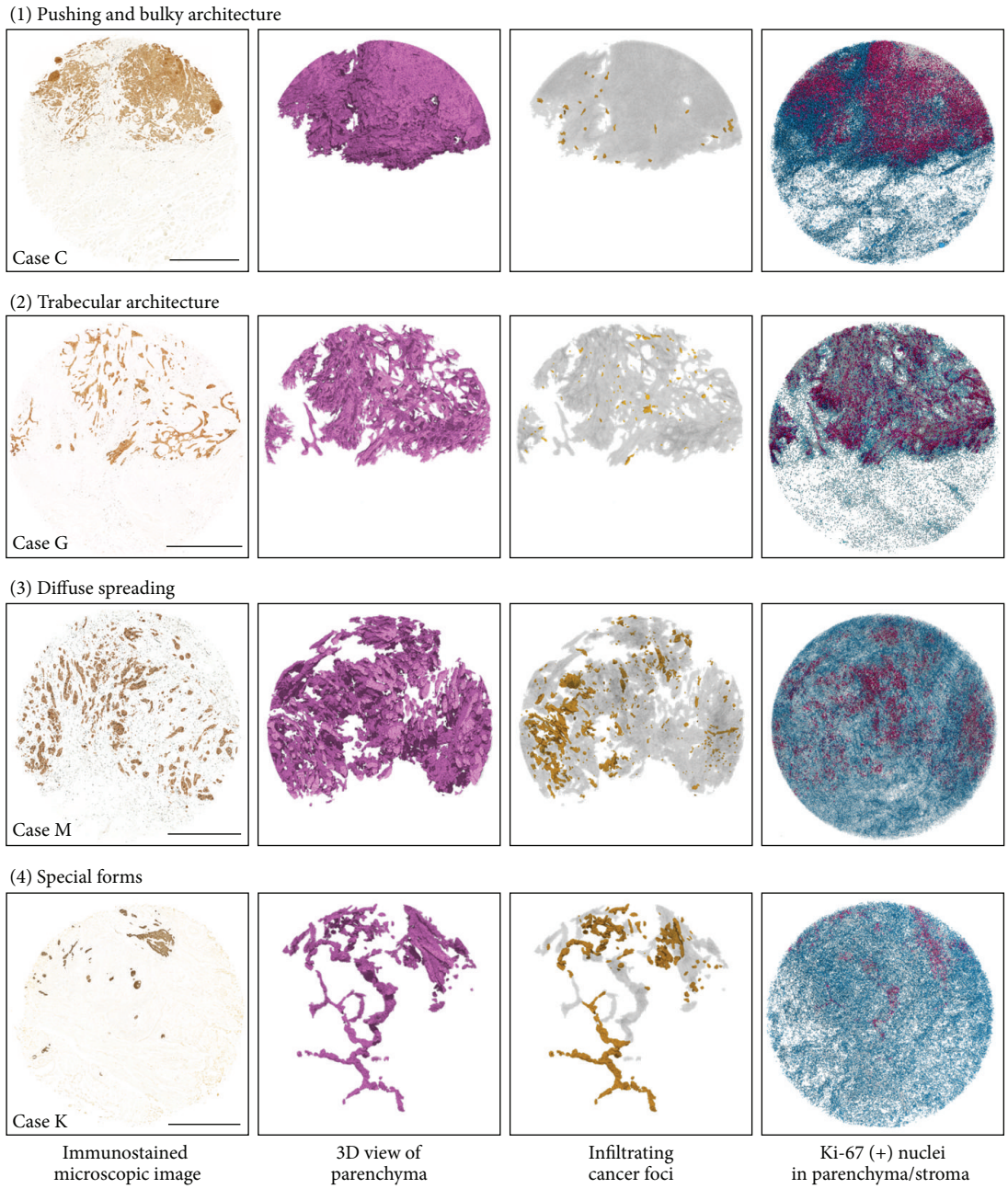

Figure 4: Four types of tumor architecture and mitotic activity at the OTSCC invasion front. Images are from left to right: the immunostained microscopic (2D) image; 3D view of the segmented tumor parenchyma; 3D view of infiltrating cancer foci (yellow) detached in all dimensions from the bulk tumor parenchyma (gray); and Ki67-positive nuclei in the tumor parenchyma (red) and stroma (blue). Additional 3D data obtained from the remaining 10 OTSCC cases can be found in Supplementary Material Plates S2 and S3. Bar = 1 mm.

(2) *Trabecular architecture with strands and cords*: in 2D microscopic images acquired from this type of SCC case, cancer cells were infiltrating diffusely in various forms of extended tortuous cords and strands, which in part gave rise to an interconnected alveolar appearance. The 3D reconstruction verified the continuity of most cords and strands, which generated a honeycomb architecture that permeated through an extensive stromal volume. Discohesive cancer foci were also localized along the trabecular tumor mass, usually with no evidence of distant migration into the host stromal environment. The Ki67-positive nuclei were distributed randomly and homogeneously throughout the tumor trabeculae reflecting the close spatial contract between the cancer cells and their adjacent stromal environment.

(3) *Diffuse spreading*: on the 2D images, this type of tumor invasion was characterized by a scattering of small cancer foci or cells in the muscular structure of the tongue, a finding substantiated by 3D analysis,

Table 3: Size distribution of discohesive cancer foci segmented at the invasion front.

OTSCC cases[1]	A	B	C	D	E	F	G	H	I	J	K	L	M	N	Total
Depth of invasion (mm)	1.9	2.4	2.5	2.6	2.8	2.9	3.3	3.5	3.6	4.6	7.0	7.6	10.6	ND	
Invasion mode	DS	PB	PB	PB	TSC	PB	TSC	PB	TSC	TSC	SP	DS	DS	SP	
Occult metastasis	−	+	+	−	−	+	−	+	−	+	+	+	+	−	
Size (μm)[2]	Numbers of discohesive cancer foci														
16>	4	0	0	12	1	13	4	2	0	2	1	17	18	1	75
16–20	32	1	0	29	12	73	33	5	0	13	15	80	63	23	379
21–25	18	1	13	8	3	21	14	7	0	10	21	54	32	5	207
26–30	5	0	12	2	1	4	10	3	1	7	23	33	19	2	122
31–35	3	0	4	1	0	1	5	1	2	2	12	22	8	1	62
36–40	0	0	1	0	1	2	2	0	0	1	11	9	3	0	30
41–45	1	0	0	0	0	1	1	1	1	0	10	6	7	1	29
46–50	1	0	0	1	1	1	7	0	3	1	21	11	8	2	57
51–200	1	0	0	0	0	0	0	0	0	0	3	0	1	0	5
200<	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	65	2	30	53	19	116	76	19	7	36	117	232	159	35	966

[1] Among the clinicopathological features of 14 OTSCC cases, most relevant terms are relisted here (see Tables 1 and 2 for the details).
[2] The size of the segmented discohesive cancer foci is expressed in terms of diameter of a sphere of the same volume.

which verified the presence of many discohesive cancer foci consisting of single or a few cancer cells, as shown below. It is notable that mitotic activity was not prominent in these infiltrating cancer cells, but was extended widely into the host environment rather than being localized at the tumor margin.

(4) *Special forms*: on the 2D images, this type of tumor invasion featured localized infiltration of only a small number of cancer cells or clumps into the deep tongue musculature. The 3D segmentation confirmed that this punctate appearance corresponded to a cross-sectional view of slender, tortuous strands, and cords extending into the intermuscular space without massive destruction of the muscular structure of the tongue. In accordance with their limited destructive capacity, the distribution of Ki67-positive nuclei through the tumor architecture was sparse and heterogeneous in the tortuous strands and cords.

3.3. Morphometric Features of 3D Tumor Architecture at the Invasion Front. Table 2 gives the results of 3D morphometric analyses of 14 OTSCC cases studied. These OTSCC cases, labeled A–N, are listed in order of the measured depth of infiltration. By initially dividing these cases into three classes (shallow, <3 mm infiltration; intermediate, 3–5 mm; and deep, >5 mm), we found that four out of the five OTSCC cases with "pushing and bulky architecture" belonged to the shallow class (the other being in the intermediate class), while three out of four cases with "trabecular architecture" were in the intermediate class (the other being in the shallow class). All of the "diffuse spreading" and "special forms" cases belonged to the deep class, except for one case that featured diffuse spreading but only shallow infiltration.

Quantitative analyses showed several unique features of OTSCC cases depending on their 3D architecture. The reconstructed tissue volume ranged from 2.19 to 2.95 mm^3, depending on the number of serial sections used. The volume of segmented tumor parenchyma varied widely from 1 to 42% of total tissue volume; as expected, the volume values were generally higher in the "pushing and bulky architecture" group and the lowest in the "special forms" group. In accordance with the intricate tumor-host border as depicted in Figure 3, the values of border area also varied widely, from 3.8 to 81.8 mm^2. By normalizing the border area (S) against the tumor parenchyma volume (V_p), it is notable that five OTSCC cases in the "pushing and bulky architecture" group yielded relatively uniform values (average $S/V_p = 78.6 \pm 18.2\ mm^{-1}$), considerably lower than the average S/V_p values for four cases of "trabecular architecture" ($81.6 \pm 45.5\ mm^{-1}$) and three cases of "diffuse spreading" ($136.4 \pm 40.6\ mm^{-1}$). The spatial resolution of the 3D reconstruction was sufficient to determine 10^3–10^5 Ki67-positive nuclei in the tumor parenchyma of each sample. By normalizing the number of Ki67-positive nuclei against tumor parenchyma volume, we revealed 6-fold differences in the average mitotic activity between OTSCC cases, although there were no coherent architecture-dependent trends. Table 2 also shows the number of discohesive cancer foci. These varied from 2 to 232 in number and, when normalized as a percentage of the tumor parenchyma volume, it was ascertained that the contribution of detached cancer cells to the total tumor mass was only marginal in the "pushing and bulky architecture" group but was markedly increased in the "diffuse spreading" and "special forms" groups.

3.4. Size Distribution of Discohesive Cancer Foci Segmented at the Advancing Front. Table 3 shows the size distribution analysis of 966 discohesive cancer foci segmented at the invasive front of 14 OTSCC cases. In this analysis, the volume of each segmented cancer mass was determined in the reconstructed 3D space and, for the sake of simplicity, the size distribution was expressed in terms of the diameter of a sphere of

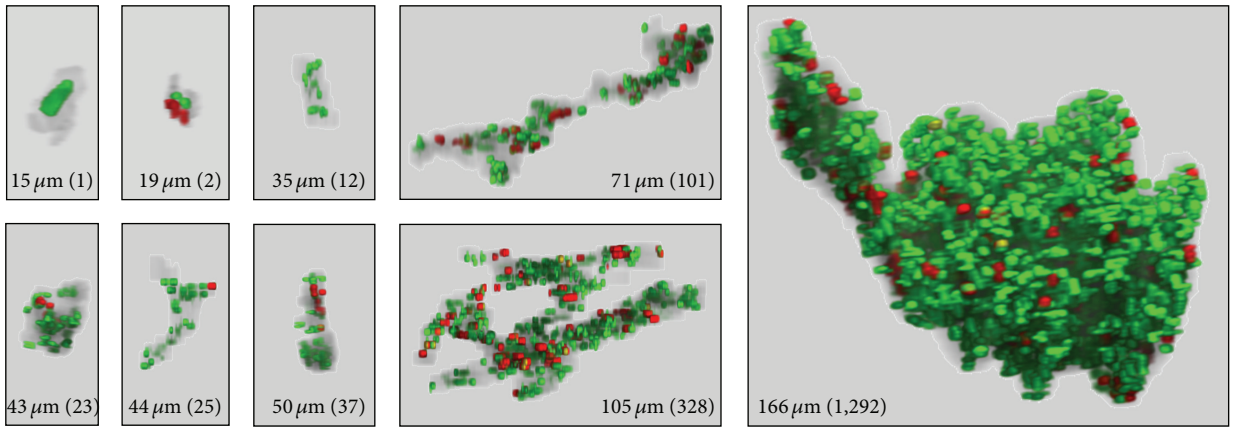

Figure 5: Three-dimensional view of individual cancer foci segmented at the invasion front. The size of the corresponding cancer mass is expressed in terms of diameter of a sphere having the same volume. The number in parentheses indicates the number of nuclei segmented from the cancer volume. Red: Ki67-positive nuclei; green: Ki67-negative nuclei (see text for nuclear segmentation).

the same volume. Notably, the majority of discohesive cancer foci were small in size, with the greatest population (39%) having a diameter ranging between 16 to 20 μm. To gain further insight into the cellular constitution of these discohesive cancer foci, we developed a computational algorithm using Ratoc TRI-SRF2 software to distinguish Ki67-positive and Ki67-negative nuclei in individual cancer foci. Ki67-negative nuclei were designated as open space circumscribed by CK-positive cytoplasm (see Figure 2(d)); noise images were deleted in the same way as applied for segmentation of Ki67-positive nuclei. To date, we have completed 3D analyses and visualization of 50 discohesive cancer foci randomly selected from different size ranges and OTSCC cases. Figure 5 shows representative 3D images of Ki67-positive (red) and Ki67-negative (green) nuclei in discohesive cancer foci. The smallest consisted of a single CK-positive cell with a Ki67-negative nucleus, while the largest analyzed so far included a total of 1,292 nuclei, comprising 277 Ki67-positive and 1,015 Ki67-negative nuclei. It is important to note that the majority (68%) of discohesive cancer foci with diameters <25 μm contained only a few cancer cells. In addition to highlighting the differences in the number of nuclei, 3D visualization of individual discohesive cancer foci disclosed their heterogeneous morphological features, for example, spheroidal, amoeboid, branching or stretching with extension of projections into the surrounding environment.

4. Discussion

The invasion mode of OTSCC is usually assessed in 2D microscopic images. However, the actual 3D configuration of tumor invasion cannot be extrapolated on the basis of 2D examination alone. For instance, an anastomosing network of tumor strands or cords within the tissue volume may appear in 2D sections as punctate cancer cells or islands. In the present study, this type of misleading appearance was most obvious in the OTSCC cases classified as "special forms". Three-dimensional reconstruction using double immunostaining with CK and Ki67 antibodies is useful in segregating the tumor parenchyma from the surrounding stroma and to assess locoregional heterogeneity in the mitotic activity of cancer cells. The direct visualization and quantitative assessment of the tumor-host border, which cannot be extrapolated from 2D examination of histological sections, provide a new dimension in our understanding of OTSCC architecture.

In the literature, the mode or mechanism of tumor invasion and metastasis has been classified into two categories: cancer cells can disseminate as individual cells (referred to as "individual cell migration") or in the form of solid cell strands, sheets, or clusters (known as "collective migration") [27]. From a practical point of view, pathologists use "tumor budding" as a prognostic factor in various human cancers [28–34]. Tumor budding is usually defined as small cell clusters composed of less than five cells at the invasive tumor margin [28, 31]. According to Bryne's malignancy grading system of oral cancer, invasive tumor islands were subdivided into two classes: >15 cells and <15 cells per island [17]. In the present study, we identified 966 discohesive cancer foci at the invasive front of 14 OTSCC cases. At present, our 3D data remain limited with respect to the morphology and cellular constitution of these foci because segmentation and morphometry of individual foci are time-consuming and labor-intensive tasks. However, the results obtained so far support the theory that both individual-cell and collective migration processes occur at the OTSCC invasion front and that the majority of discohesive cancer foci comprise only a few cancer cells.

In relation to the mechanism of detachment of carcinoma cells from the primary tumor mass, it is widely accepted that these alter their phenotypes with the progression of malignancy, resulting in a loss of cell attachment and/or an epithelial-mesenchymal transition (EMT) [35, 36]. Regarding the possibility of EMT in OTSCC at the invasion front, it should be noted that our RGB color segmentation depended on the immunoreactivity of cancer cells for a cocktail of anti-CK antibodies, so that segmented carcinoma cells undergoing single-cell invasion retain their epithelial phenotype without experiencing a complete EMT. Obviously, it is important to

characterize the phenotypic changes that accompany the initiation and progression of discohesive invasion of SCC cells, and this future work could investigate additional putative immunolabels such as E-cadherin, vimentin, and Snail.

As described in the Introduction, the clinico-pathological predictors of outcome in patients with early stage OTSCC have been intensely studied. The critical depth of infiltration of the primary tumor in connection with nodal metastasis has been estimated to be around 4 mm [5, 9, 10]. In the present study, we examined 14 cases of OTSCC showing a range of infiltration depths from 1.9 to 10.6 mm. We first investigated whether the depth of infiltration significantly predicted occult neck metastases but rejected this hypothesis on the basis that two patients with shallow infiltration (2.4 and 2.5 mm) had occult metastasis. Next, we critically evaluated the relationship between the frequency of discohesive cancer foci and the depth of infiltration. Table 3 shows that the frequency and size of discohesive cancer foci increase with the depth of infiltration; this trend was most prominent in the deep-infiltrating OTSCC cases with diffuse spreading and special forms. Of more importance to pathological diagnosis, however, are the findings that detachment of a single or a few cancer cells occurred in the shallow infiltration groups regardless of the type of tumor architecture and that occult neck metastasis was associated with all types of 3D tumor architecture and invasion modes, as exemplified in case B, which had a delineated pushing border and only two discohesive cancer foci in the analyzed 3D space. Although the mechanisms underlying the capacity of shallow-infiltrating OTSCCs to metastasize via lymphatic vessels have not yet been fully elucidated, it is pertinent to point out that even a massive OTSCC with a pushing border possessed a wide surface area in contact with the stromal environment, including blood/lymph vessels. Ohno et al. [37] previously reported that OTSCC cells in clumps contact pre-existing dilated lymphatic vessels and break through the thin-walled lymphatic vessel to enter the lumen. In our seminal 3D reconstruction in combination with immunolabels of tumor and vascular endothelial cells, we also observed that the tips of the primary tumor mass of an OTSCC frequently penetrate through thin-walled lymphatic vessels to enter the lumen, resulting in tumor emboli in the lumen. Taken together, we consider that dissemination of cancer cells via the lymphatic vessels may occur at very early stages of OTSCC development without the manifestation of metastatic growth in new environments.

5. Conclusion

The development of new technologies and methods is continually increasing the speed and utility of histology-based 3D reconstruction for investigating various anatomical and pathological objects [24, 26]. The present 3D image reconstruction proved the feasibility of volumetric isolation of OTSCC architecture segmented from the surrounding stroma at high spatial resolution. There is still a paucity of information regarding the mechanism of dissemination of OTSCC leading to regional lymph node and distant metastases. It will be necessary to visualize the spatial proximity of the tumor architecture and the vasculature and lymphatics in the tumor-host environment, an investigation currently ongoing in our laboratory.

Conflict of Interests

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this paper.

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Clinical Study

Immunohistochemical Analysis of the Activation Status of the Akt/mTOR/pS6 Signaling Pathway in Oral Lichen Planus

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Introduction. Aberrations of the Akt/mTOR/pS6 pathway have been linked to various types of human cancer, including oral squamous cell carcinoma (OSCC). The purpose of this study was to evaluate the activation status of Akt, mTOR, and pS6 in oral lichen planus (OLP) in comparison with oral premalignant and malignant lesions and normal oral mucosa (NM). *Materials and Methods*. Immunohistochemistry for p-Akt, p-mTOR, and phospho-pS6 was performed in 40 OLP, 20 oral leukoplakias (OL), 10 OSCC, and 10 control samples of NM. *Results*. Nuclear p-Akt expression was detected in the vast majority of cases in all categories, being significantly higher in OL. Cytoplasmic p-Akt and p-mTOR staining was present only in a minority of OLP cases, being significantly lower compared to OL and OSCC. Phospho-pS6 showed cytoplasmic positivity in most OLP cases, which however was significantly lower compared to OL and OSCC. *Conclusions*. Overall, cytoplasmic p-Akt, p-mTOR, and phospho-pS6 levels appear to be significantly lower in OLP compared to OL and OSCC. However, the expression of these molecules in a subset of OLP cases suggests that activation of Akt/mTOR/pS6 may occur in the context of OLP, possibly contributing to the premalignant potential of individual cases.

1. Introduction

Oral lichen planus (OLP) is an immunologically mediated disease, the malignant potential of which has been a subject of intense investigation and ongoing controversy [1, 2]. Several published series of OLP with long-term follow-up have reported a variable rate of malignant transformation ranging from 0 to 12.5%, with most authorities suggesting that the actual percentage revolves around 1% [1, 2]. Although the overall malignant transformation rate appears low, the increased frequency of OLP in the general population makes it necessary to investigate the epidemiologic, clinical, and histopathologic factors related to the premalignant nature of OLP and also to elucidate the possible molecular mechanisms and pathways underlying oral carcinogenesis in the context of a preexisting OLP lesion [3].

In recent years, advances in molecular biology have allowed the dissection of several carcinogenesis-related signaling pathways and have offered insight into the molecular events and aberrations mediating cancer development and progression. Akt/mTOR/pS6 signaling has been identified as one of the most commonly implicated pathways in various types of human cancer, including oral squamous cell carcinoma [4, 5]. Akt is a serine-threonine kinase that functions as a downstream target and effector of phosphatidylinositol 3-kinase (PI3K). It is frequently activated in human cancers and precancerous lesions and is considered a key regulator of normal and cancerous cell growth and fate decisions [6, 7]. mTOR is one of the major targets of activated Akt, which in turn regulates a number of downstream molecules, such as ribosomal protein pS6, eventually controlling fundamental cell processes such as cell survival, proliferation, protein synthesis, and angiogenesis [4, 6, 7]. Dysregulations in upstream and downstream molecules of mTOR signaling appear to occur in 90–100% of HNSCC suggesting that markers and targets in the Akt/mTOR/pS6 pathway may be of particular clinical relevance [8].

The purpose of this study was to assess the immunohistochemical expression of the phosphorylated (activated) forms of Akt, mTOR, and pS6 in biopsy samples of OLP in

comparison with oral leukoplakia (OL) with various degrees of dysplasia, oral squamous cell carcinoma (OSCC), and control cases of normal mucosa (NM), in order to evaluate the potential contribution of Akt/mTOR/pS6 signaling pathway aberrations in OLP malignant potential.

2. Materials and Methods

2.1. Patients and Tumor Samples. Formalin-fixed, paraffin-embedded tissue samples from 40 patients diagnosed with OLP, 20 patients diagnosed with OL (including 5 hyperplasias, 5 mild dysplasias, 5 moderate dysplasias, and 5 severe dysplasias), and 10 patients diagnosed with OSCC at the Department of Oral Medicine and Pathology, University of Athens, Greece, between 2005 and 2008 were identified. Ten control cases of oral NM of healthy subjects were also included. Clinical records for these patients were reviewed, and information regarding the final diagnosis was obtained. Representative hematoxylin and eosin sections of each tumor were reviewed, and the diagnosis (as well as degree of dysplasia for OL and grade of differentiation for OSCC) was confirmed according to well-accepted diagnostic criteria [9].

2.2. Immunohistochemistry. Five micron-thick serial sections of formalin-fixed and paraffin-embedded tissues were immunostained in the Leica BOND-MAX fully automated immunohistochemistry system (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), by applying the NovoLink Polymer Detection System (Leica Biosystems Newcastle Ltd). For epitope retrieval, a high-temperature technique with citrate buffer was utilized. The sections were incubated in 3% hydrogen peroxide (Novocastra Peroxidase Block, Novocastra Leica Microsystems) to neutralize endogenous peroxidase activity; treated with Novocastra Protein Block to reduce nonspecific binding of primary antibody and polymer; incubated with primary antibodies; and treated with Novocastra Postprimary Block, containing 10% (v/v) animal serum in tris-buffered saline, to enhance penetration of the subsequent polymer reagent. Consequently, poly-HRP anti-mouse/rabbit IgG reagent (NovoLink Polymer) containing 10% (v/v) animal serum in tris-buffered saline was applied to localize the primary antibody, and the reaction product was visualized by incubation with the substrate/chromogen, 3,3′-diaminobenzidine (DAB) prepared from Novocastra DAB Chromogen and NovoLink DAB Substrate Buffer (Polymer), as a brown precipitate. Finally, the sections were counterstained with Novocastra Hematoxylin (0.02%).

The following primary antibodies were used: rabbit monoclonal antibody against phosphorylated Akt (1 : 100, p-Akt, phosphorylated at serine 473, Cell Signaling Technology Inc., no. 4060); rabbit monoclonal antibody against phosphorylated mTOR (1 : 100, p-mTOR, phosphorylated at serine 2448, Cell Signaling Technology Inc., no. 2976); and rabbit polyclonal antibody against phosphorylated S6 protein (1 : 100, phospho-pS6, phosphorylated at serine 235/236, Cell Signaling Technology Inc., no. 2211). Due to limited available tissue material, a few cases were not stained with all three antibodies.

Appropriate positive control cases were used for both antibodies. As a negative control, sections were treated with phosphorylated buffered saline (PBS) with omission of the primary antibody.

The immunostains were reviewed by two independent evaluators (GP and NN). The interobserver variability was very low (<5% of cases). In cases in which there was initial disagreement, stains were reevaluated by the aforementioned investigators using a multiobserver microscope and discussed until consensus was reached.

Immunohistochemical reactivity for p-Akt, p-mTOR, and phospho-pS6 stains was graded in a semiquantitative manner according to the percentage of positive epithelial cells: (0) 0%, (1) <20%, (2) 20–50%, and (3) >50%, and the intensity of staining: (0) no staining, (1) weak, (2) moderate, or (3) strong, as compared to the negative control tissues. Moreover, a combined score of immunohistochemical positivity (0, 2–6) was calculated for each case by adding the individual scores for percentage of cells (0–3) and intensity of staining (0–3). In cases of OLP, OL, and NM, positive cells were identified within the whole epithelial thickness. For OSCC, immunostaining was evaluated in the tumor cell population. The subcellular localization of immunohistochemical staining (nuclear versus cytoplasmic staining pattern) was also recorded and, where appropriate, scored separately.

2.3. Statistical Analysis. Immunohistochemical scores (intensity, positivity, and total scores for p-Akt, p-mTOR, and phospho-pS6) were compared between OLP and the other categories (OL, OSCC, and NM) using nonparametric tests (Mann-Witney). A statistically significant difference was considered to be present at $P \leq 0.01$ (following Bonferroni correction).

3. Results

3.1. p-Akt. p-Akt staining was noticed to be localized in the nucleus and/or the cytoplasm of the epithelial cells in the various cases studied (Figure 1). Because of the observed heterogeneity of p-Akt staining localization and taking into account previous studies reporting nuclear p-Akt staining in oral epithelial lesions, as well as neoplasms of diverse origin [10–15], both nuclear and cytoplasmic p-Akt immunoreactivities were analyzed.

3.1.1. Nuclear p-Akt. Of 40 OLP cases studied for nuclear expression of p-Akt, 37 (92.5%) were positive, whereas 3 (7.5%) were negative. Of the positive cases, 7 (17.5%) showed nuclear p-Akt immunopositivity in <20% of epithelial cells, while 17 (42.5%) and 13 (32.5%) cases exhibited staining in 20–50% and >50% of epithelial cells, respectively; the average score for the percentage of positive epithelial cells for nuclear p-Akt was 2.00. On the other hand, the average score for the staining intensity was 1.53, corresponding to 15 (37.5%) cases that were stained weakly, 20 (50%) moderately, and 2 (5%) strongly. The average combined score of nuclear p-Akt immunohistochemical positivity in OLP was 3.53.

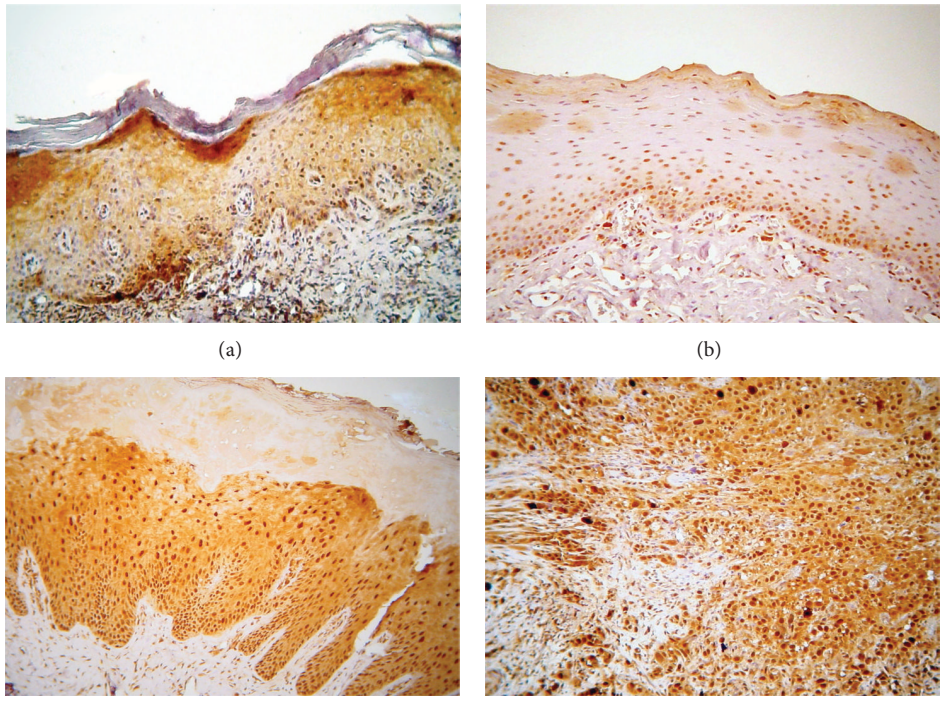


Figure 1: Immunohistochemical expression of phosphorylated Akt at serine 473 (p-Akt) in selected cases of (a) oral lichen planus (OLP), (b) normal mucosa (NM), (c) oral leukoplakia (OL), and (d) oral squamous cell carcinoma (OSCC) (immunohistochemistry, 100x magnifications).

Table 1: Percentage of positive cases and average positivity, intensity, and total scores for nuclear p-Akt per lesion category.

	Number and % of positive cases	Average positivity score	Average intensity score	Average total score
OLP	37/40 (92.5%)	2.00	1.53	3.53
NM	9/9 (100%)	1.78	1.78	3.56
OL	17/17 (100%)	2.94*	2.71*	5.65*
OSCC	7/10 (70%)	1.80	1.30	3.10

OLP: oral lichen planus; NM: normal mucosa; OL: oral leukoplakia; OSCC: oral squamous cell carcinoma.
*Statistical significant differences ($P < 0.05$), compared to OLP.

Regarding nuclear p-Akt staining in OL, all studied cases were positive. Based on the percentage of positive cells, 16 cases (94.1%) received score 3 and 1 case (5.9%) received score 2 for positivity (average score: 2.94). The average intensity score was 2.71; one case (5.9%) received score 1, 3 cases (17.6%) score 2, and 13 cases (76.5%) received score 3. The average combined score for nuclear p-Akt immunohistochemical positivity in oral leukoplakia was 5.65.

On the other hand, OSCC showed positivity in 70% of cases studied; the average positivity, intensity, and total scores were 1.8, 1.3, and 3.1, respectively. All NM cases were positive and the corresponding positivity, intensity, and total scores were 1.78, 1.78, and 3.56, respectively.

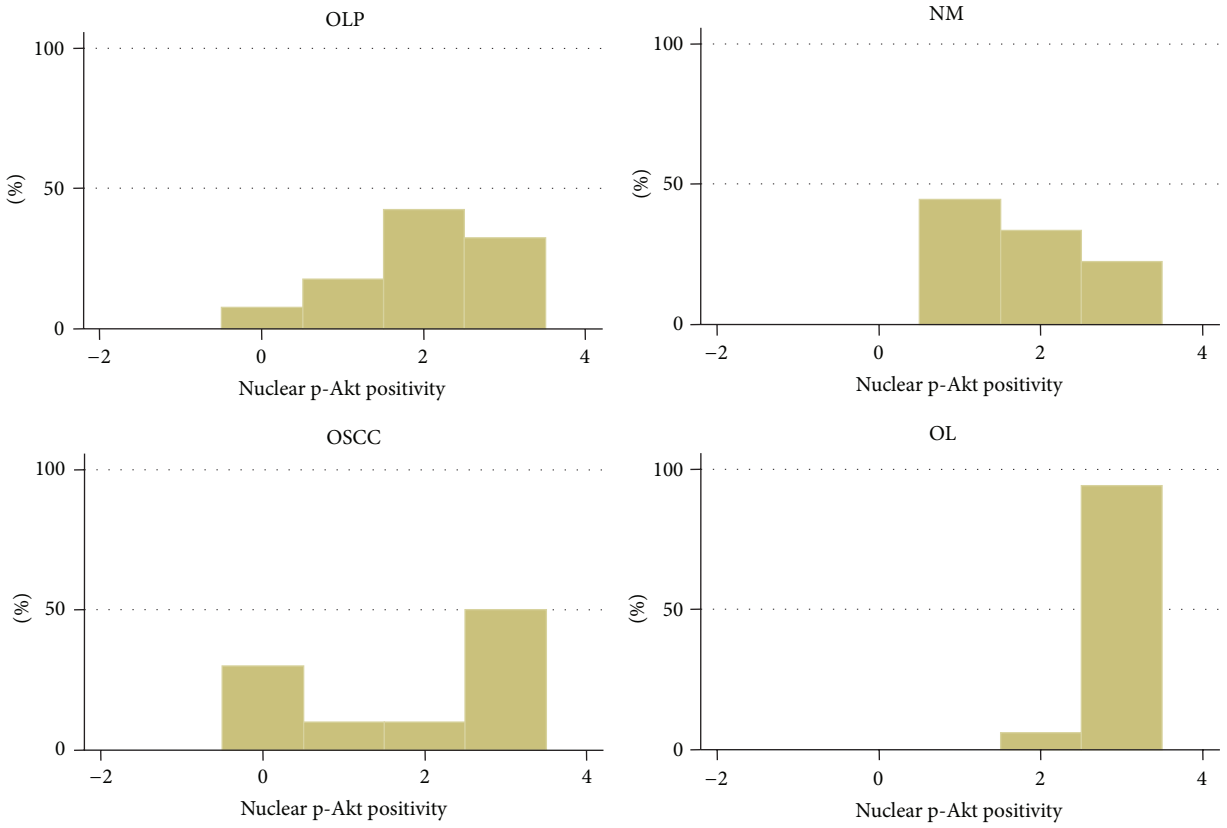
Statistical analysis revealed significant differences between OLP and OL regarding intensity ($P < 0.0001$), positivity ($P < 0.0001$), and total scores ($P < 0.0001$); in other words, OL received higher scores for p-Akt nuclear expression compared to OLP. In contrast, no significant differences between OLP and NM or between OLP and OSCC were noticed.

The results for nuclear p-Akt are summarized in Table 1 and Figure 2.

3.1.2. Cytoplasmic p-Akt. Of the 40 OLP cases studied for cytoplasmic expression of p-Akt, 38 (95%) were negative and only 2 cases (5%) were positive. The two positive cases showed weak or moderate immunostaining in less than 20% of epithelial cells, so that the average positivity, intensity, and total scores for cytoplasmic p-Akt were 0.05, 0.08, and 0.05, respectively.

With regard to OL, 6 cases (35.3%) were negative and 11 cases (64.7%) were positive. The average positivity, intensity, and total scores for cytoplasmic p-Akt in OL were 0.94, 1.06, and 2.00, respectively.

All NM cases were negative. In contrast, 70% of OSCC were positive; the average positivity, intensity, and total scores



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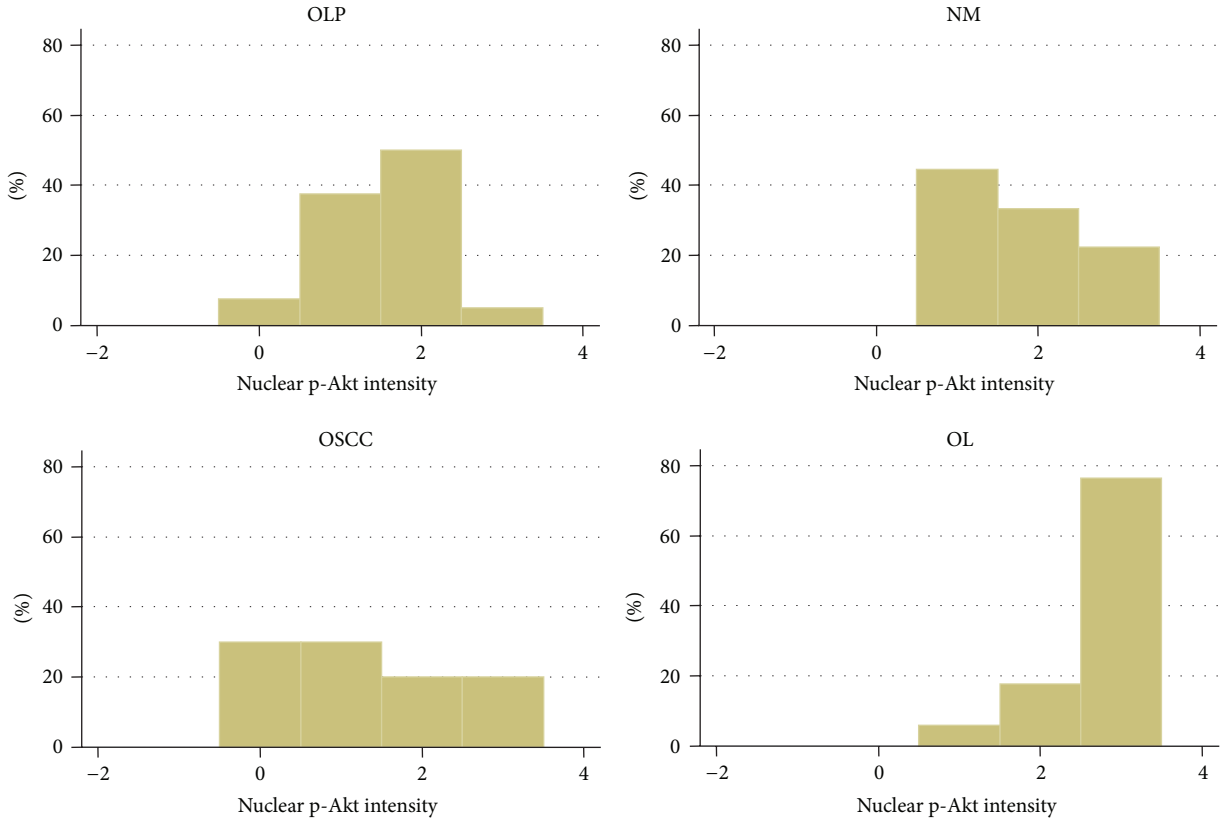

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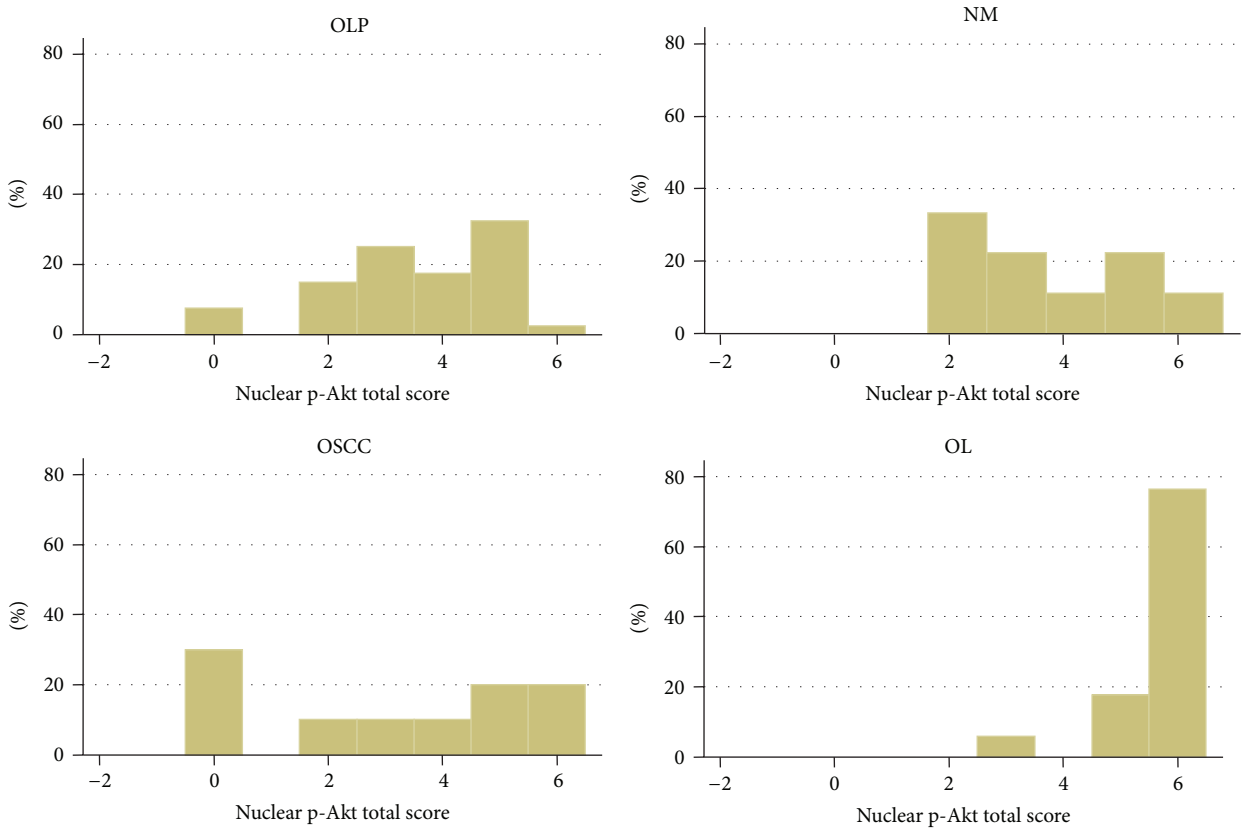

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Figure 2: Graph of immunohistochemical results for nuclear p-Akt. Distribution of cases per lesion category according to (a) positivity score, (b) intensity score, and (c) total score. Abbreviations: OLP: oral lichen planus; NM: normal mucosa; OSCC: oral squamous cell carcinoma; OL: oral leukoplakia.

Table 2: Percentage of positive cases and average positivity, intensity, and total scores for cytoplasmic p-Akt per lesion category.

	Number and % of positive cases	Average positivity score	Average intensity score	Average total score
OLP	2/40 (5%)	0.05	0.08	0.05
NM	0/9 (0%)	0	0	0
OL	11/17 (64.7%)	0.94*	1.06*	2.00*
OSCC	7/10 (70%)	1.10*	1.10*	2.20*

OLP: oral lichen planus; NM: normal mucosa; OL: oral leukoplakia; OSCC: oral squamous cell carcinoma.
*Statistical significant differences ($P < 0.05$), compared to OLP.

for cytoplasmic p-Akt in oral SCC were 1.10, 1.10, and 2.20, respectively.

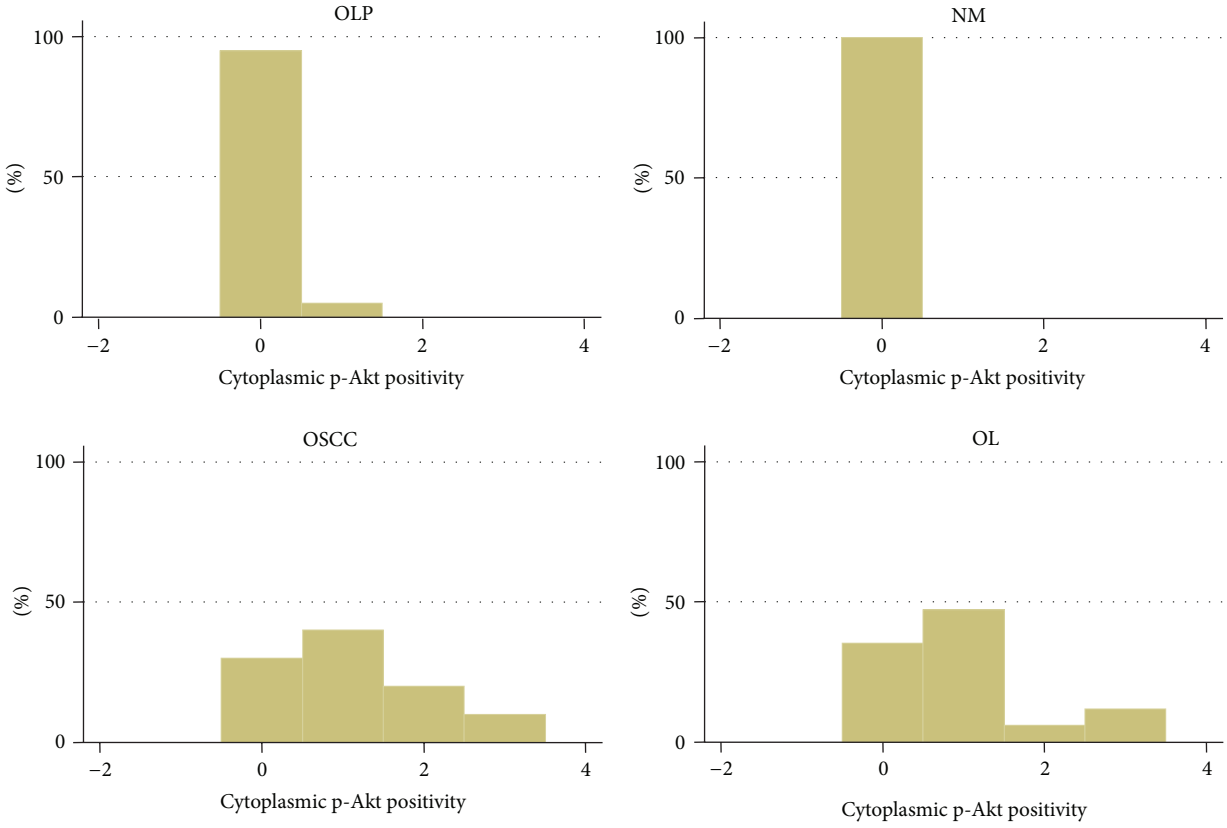
Statistical analysis showed no significant differences between OLP and NM. However, the intensity, positivity, and total scores for p-Akt cytoplasmic expression were significantly lower in OLP compared to both OL ($P < 0.0001$) and OSCC ($P < 0.0001$).

The results for cytoplasmic p-Akt are summarized in Table 2 and Figure 3.

3.2. p-mTOR. p-mTOR was expressed in a subset of the cases studied showing exclusive cytoplasmic localization (with the exception of a single case of OLP showing nuclear expression) (Figure 4).

Of the 39 OLP cases studied for immunohistochemical expression of p-mTOR, 35 (89.7%) were negative. Only 4 cases (10.3%) were positive, corresponding to 3 cases (7.7%) with immunoreactivity in <20% of epithelial cells and 1 case (2.6%) showing staining in >50% of epithelial cells. The average positivity, intensity, and total scores for p-mTOR in OLP were 0.154, 0.154, and 0.308, respectively.

Regarding p-mTOR staining in OL, 7 cases (36.8%) were negative, and 12 cases (63.2%) were positive. More specifically, 5 cases (26.4%) received score 1 and 7 cases (36.8%) received score 2 for positivity (average score: 1.00). The average intensity score was 1.05, with 5 cases (26.4%) receiving score 1, 6 cases (31.6%) receiving score 2, and one case (5.3%) receiving score 3. The average total score of p-mTOR immunohistochemical positivity in OL was 2.05.



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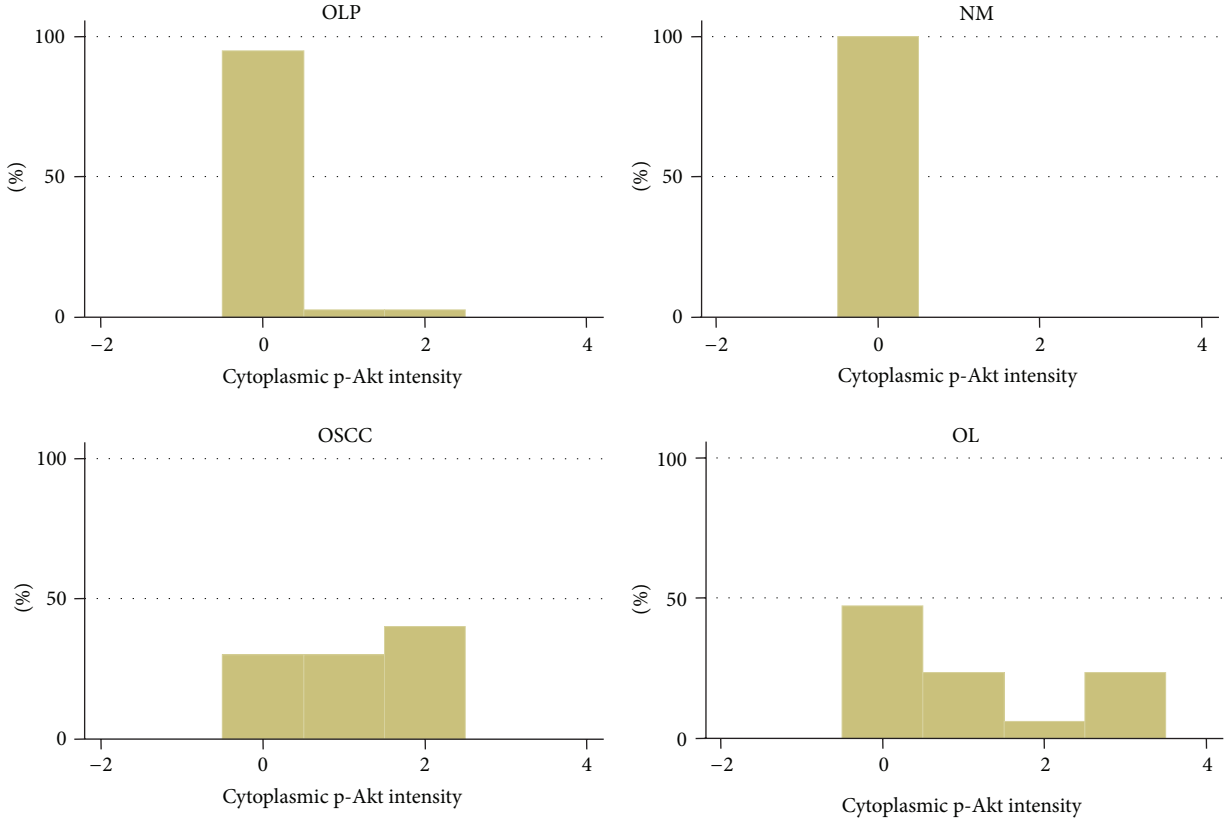

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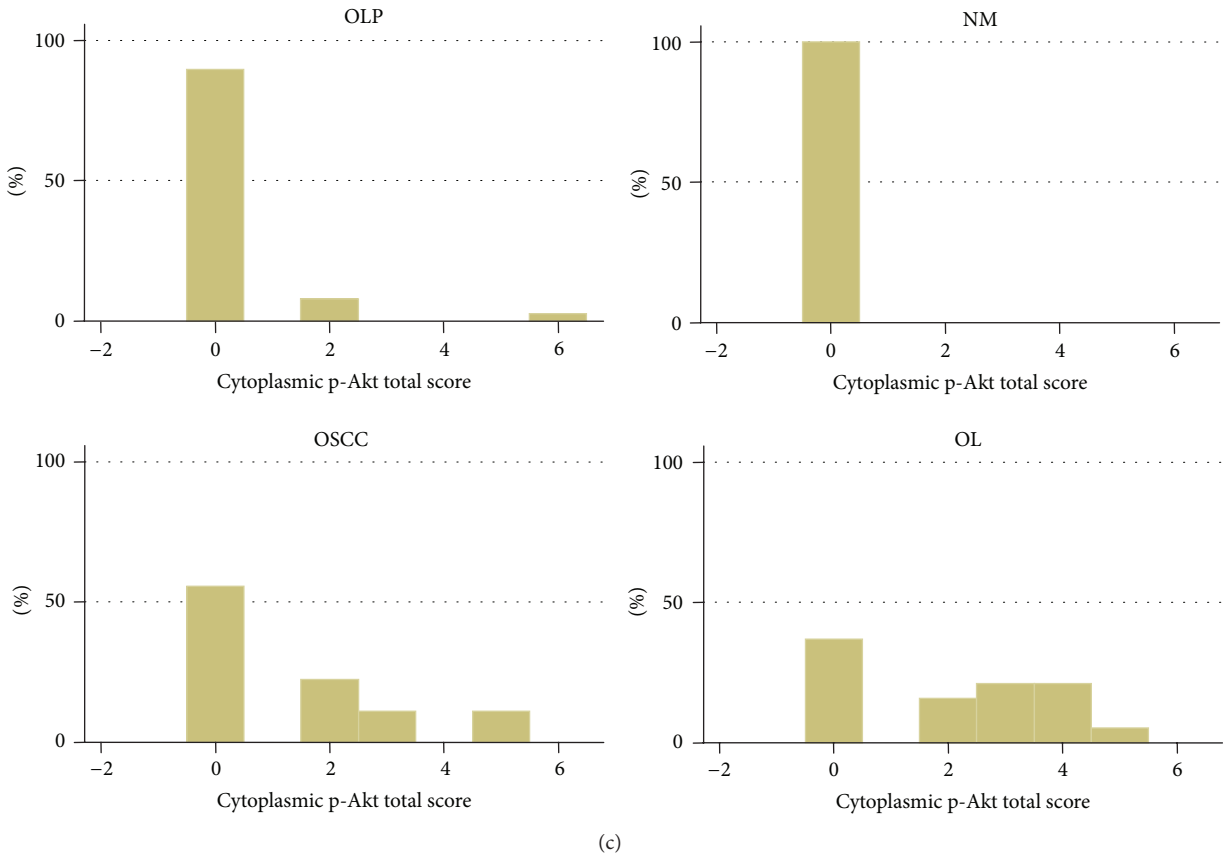


Figure 3: Graph of immunohistochemical results for cytoplasmic p-Akt. Distribution of cases per lesion category according to (a) positivity score, (b) intensity score, and (c) total score. Abbreviations: OLP: oral lichen planus; NM: normal mucosa; OSCC: oral squamous cell carcinoma; OL: oral leukoplakia.

Table 3: Percentage of positive cases and average positivity, intensity, and total scores for p-mTOR per lesion category.

	Number and % of positive cases	Average positivity score	Average intensity score	Average total score
OLP	4/39 (10.3%)	0.154	0.154	0.31
NM	0/10 (0%)	0	0	0
OL	12/19 (63.2%)	1.00*	1.05*	2.05*
OSCC	4/9 (44.4%)	0.78*	0.56*	1.33*

OLP: oral lichen planus; NM: normal mucosa; OL: oral leukoplakia; OSCC: oral squamous cell carcinoma.
*Statistical significant differences ($P < 0.05$), compared to OLP.

Of 9 studied OSCC cases, 5 (55.6%) were negative and 4 (44.4%) were positive. The average positivity, intensity, and total scores for p-mTOR in OSCC were 0.78, 0.56, and 1.33, respectively. In contrast, all NM cases were negative (average score 0).

Statistical analysis showed that no significant differences existed between OLP and NM (despite the fact that all NM cases were negative as opposed to 10.3% of OLP cases being positive). On the other hand, the intensity, positivity, and total scores for p-mTOR immunohistochemical expression were significantly lower in OLP compared to both OL ($P < 0.0001$) and OSCC ($P < 0.01$).

The results for p-mTOR are summarized in Table 3 and Figure 5.

3.3. Phospho-pS6. Phosphorylated pS6 (phospho-pS6) was immunohistochemically detected in the majority of the cases studied; the staining pattern was cytoplasmic (Figure 6).

Out of the 40 OLP cases, 36 (90%) were positive, while only 4 (10%) were negative. Most positive cases (24/40, 60%) showed immunopositivity in <20% of epithelial cells, while 9 (22.5%) and 3 (7.5%) showed staining in 20–50% and >50% of epithelial cells, respectively; the average score for percentage of positive epithelial cells for phospho-pS6 in OLP was 1.28. Regarding phospho-pS6 staining intensity, 21/40 (52.5%) cases were weak, while 15/40 (37.5%) were moderate; the average score for staining intensity was 1.28. Finally, the average combined score of phospho-pS6 immunostaining in OLP was 2.52.

Table 4: Percentage of positive cases and average positivity, intensity, and total scores for phospho-pS6 per lesion category.

	Number and % of positive cases	Average positivity score	Average intensity score	Average total score
OLP	36/40 (90%)	1.28	1.28	2.52
NM	9/9 (100%)	1.78	1.78	3.56
OL	16/16 (100%)	2.25*	1.81	4.06*
OSCC	8/9 (88.9%)	2.33*	2.33*	4.67*

OLP: oral lichen planus; NM: normal mucosa; OL: oral leukoplakia; OSCC: oral squamous cell carcinoma.
*Statistical significant differences ($P < 0.05$), compared to OLP.

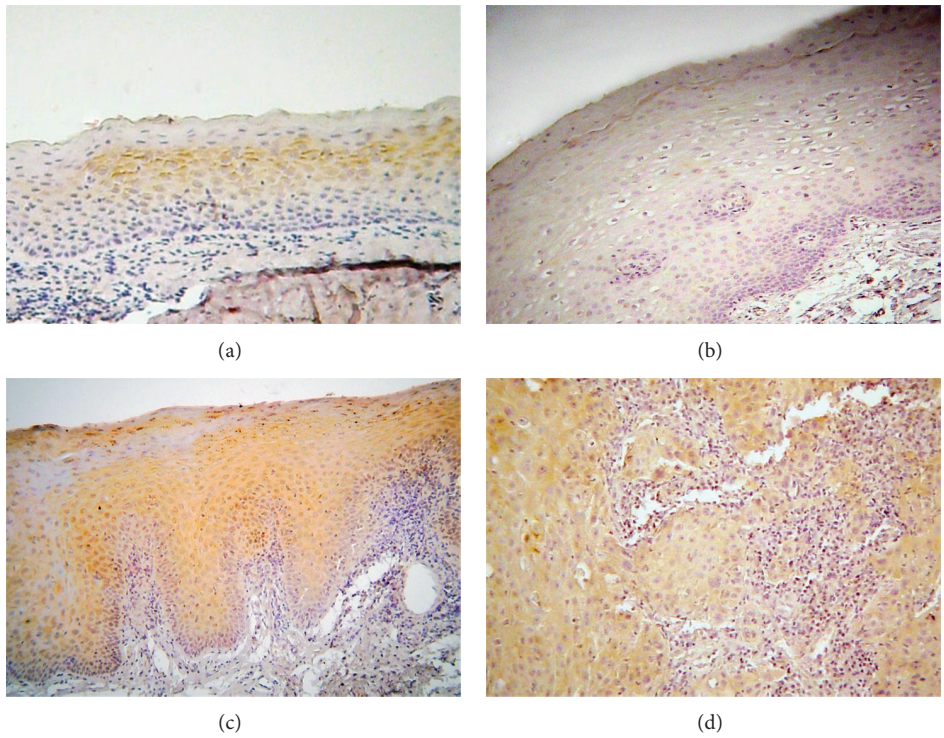


Figure 4: Immunohistochemical expression of phosphorylated mTOR (p-mTOR) in selected cases of (a) oral lichen planus (OLP), (b) normal mucosa (NM), (c) oral leukoplakia (OL), and (d) oral squamous cell carcinoma (OSCC) (immunohistochemistry, 100x magnifications).

All OL cases studied were positive for phospho-pS6 (16/16, 100%). Eight cases (50%) demonstrated immunoreactivity in 20–50% of epithelial cells, 2 cases (12.5%) were positive in <20% of cells, while 6 cases (37.5%) showed positivity in >50% of cells; the average positivity score was 2.25. On the other hand, the average intensity score was 1.81 corresponding to 8 cases (50%) receiving score 1, 3 cases (18.75%) receiving score 2, and 5 cases (31.25%) receiving score 3. The average total immunohistochemical score for phospho-pS6 in OL was 4.06.

Eight out of 9 OSCC cases (88.9%) were positive, most of them (7/9, 77.7%) showing strong immunoreactivity in >50% of tumors cells. The average positivity, intensity, and total scores for phospho-pS6 in OSCC were 2.33, 2.33, and 4.67, respectively. Finally, all NM cases were positive and the corresponding positivity, intensity, and total scores were 1.78, 1.78, and 3.56, respectively.

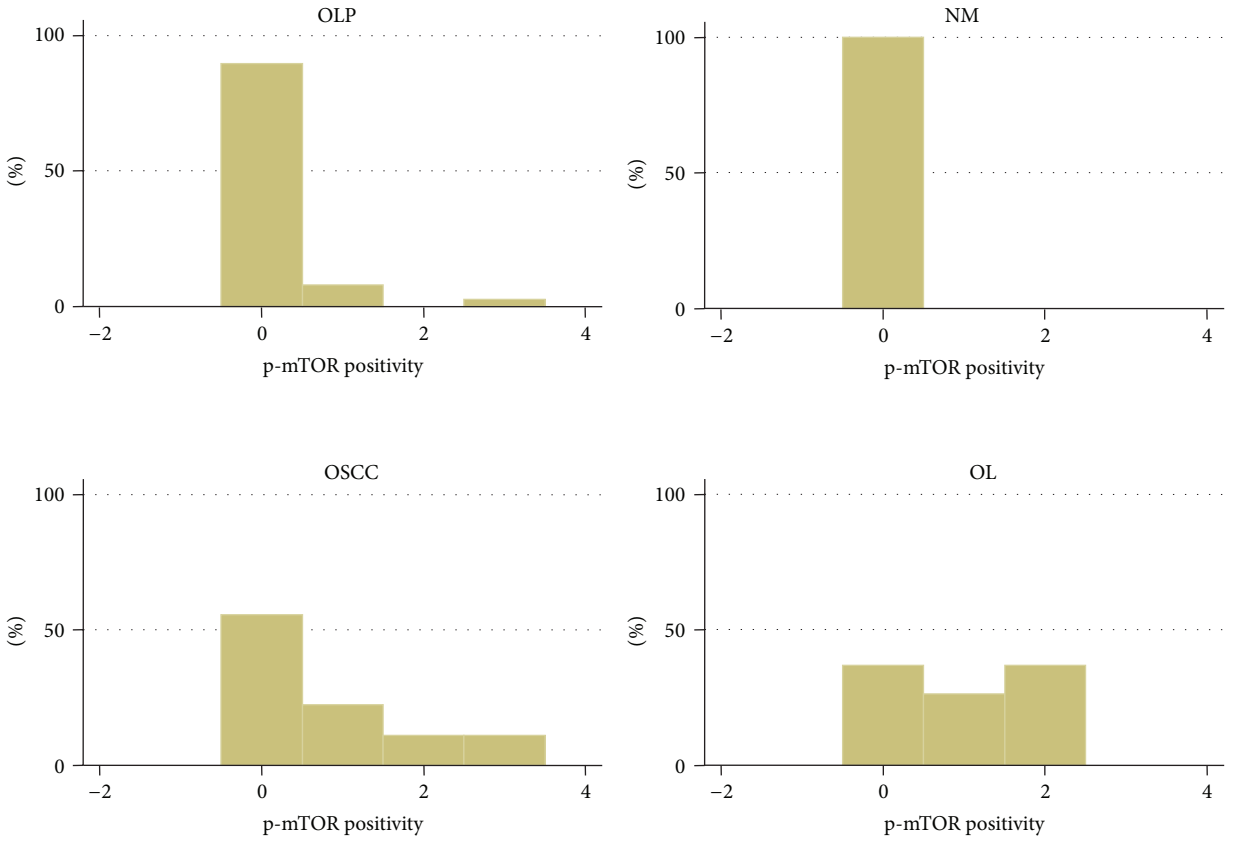
Statistical analysis did not reveal significant differences in phospho-pS6 immunoreactivity between OLP and NM. However, the intensity, positivity, and total scores for phosphor-pS6 expression were significantly lower in OLP compared to both OL ($P < 0.0004$) and OSCC ($P < 0.002$).

The results for p-mTOR are summarized in Table 4 and Figure 7.

4. Discussion

The present study attempted to investigate the activation status of the Akt/mTOR/pS6 pathway in cases of OLP compared to cancerous (OSCC) and precancerous (OL) lesions and normal oral mucosa (NM) samples. Since phosphorylation of Akt, mTOR, and pS6 is necessary for their activation, the phosphorylated levels of these molecules were examined.

To evaluate Akt activation status, an antibody recognizing Akt phosphorylated at serine 473 was employed. It has been demonstrated that Akt activation involves interaction of its N-terminal pleckstrin homology (PH) domain with



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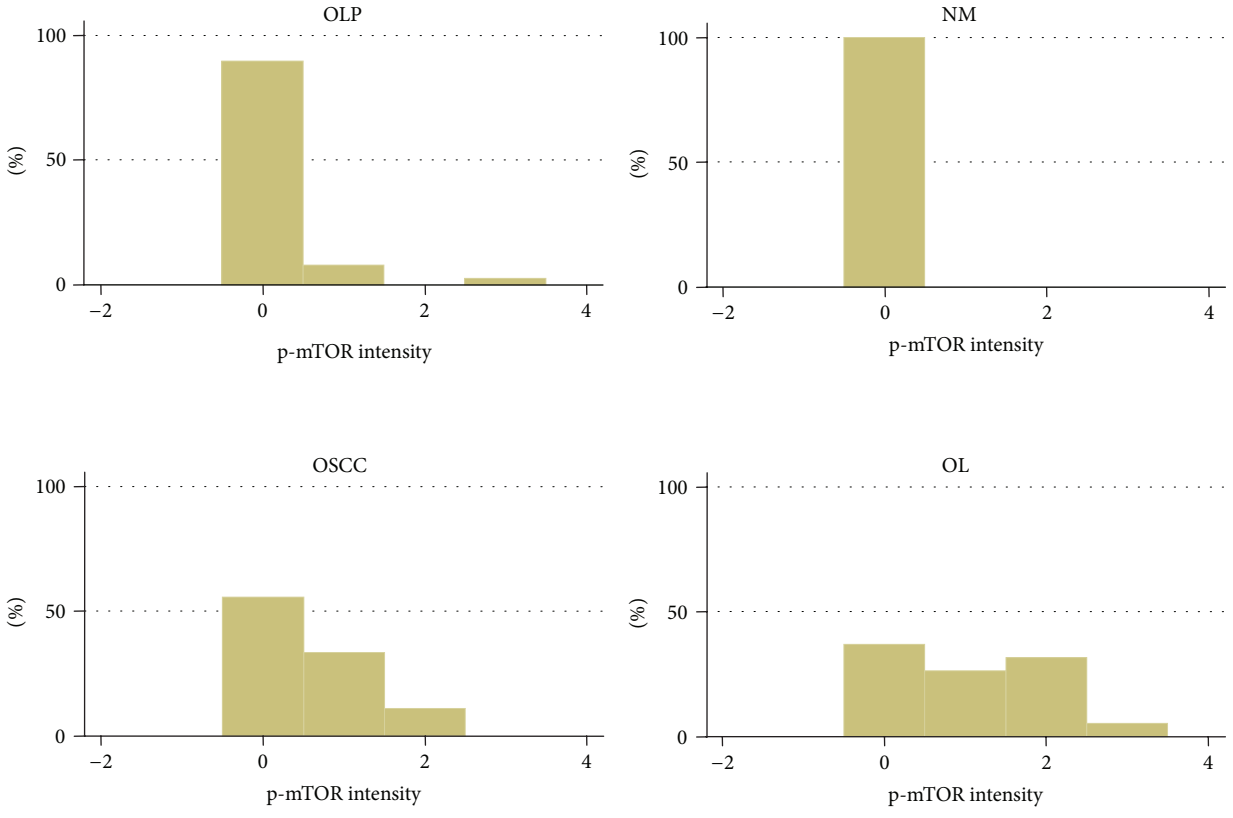

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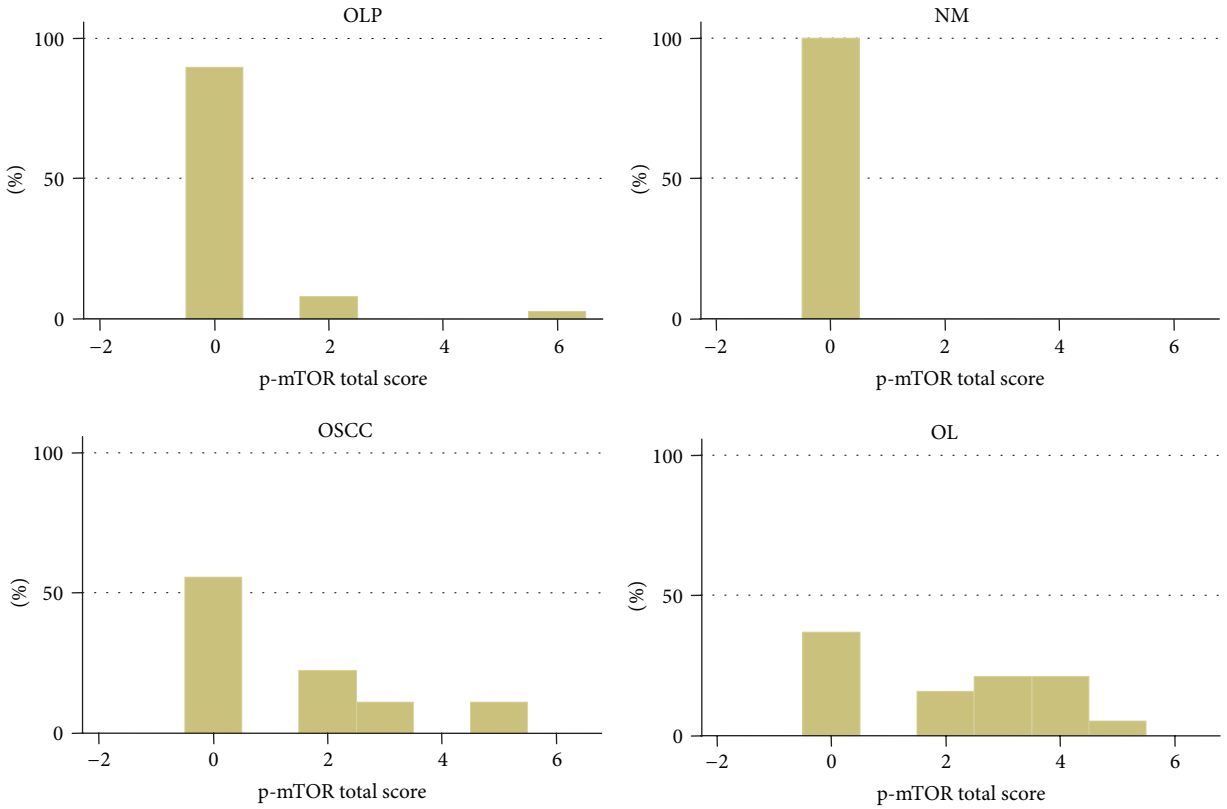


Figure 5: Graph of immunohistochemical results for p-mTOR. Distribution of cases per lesion category according to (a) positivity score, (b) intensity score, and (c) total score. Abbreviations: OLP: oral lichen planus; NM: normal mucosa; OSCC: oral squamous cell carcinoma; OL: Oral leukoplakia.

3-phosphoinositides generated by the phosphoinositide 3-kinase (PI3K) with ensuing translocation of the molecule to the plasma membrane [12, 13]. Full Akt activation requires phosphorylation by PDK1 at Thr-308 and by PDK2 at Ser-473 [16, 17]. Since Akt phosphorylation at Ser-473 involves an mTOR- containing protein complex (mTORC2), it is also a marker of mTOR activity [12, 18]. On the other hand, PTEN (phosphatase and tensin homolog deleted on chromosome 10), a well-recognized tumor suppressor gene, is a negative regulator of Akt [19, 20]. Activated Akt dissociates from the plasma membrane and exerts its activity by phosphorylating both cytoplasmic and nuclear downstream effectors, including mTOR [15]. By doing so, Akt regulates a number of critical biological functions, such as cell growth and survival, apoptosis, and metabolism. In addition, Akt activation has been associated with cancer development and progression in various human cancers and has been proposed as a potential molecular marker of prognostic and therapeutic significance [4–6].

In the present study, both nuclear and cytoplasmic levels of p-Akt were evaluated. Although the mechanisms and the significance of this compartmentalization are largely unknown, previous investigations have shown variable distribution of p-Akt in different cellular compartments of oral premalignant and malignant lesions indicating that p-Akt localization may be related to differences in activity [10, 11]. In addition, differential subcellular (nuclear vs. cytoplasmic) p-Akt localization has been reported in other types of cancer and, as suggested, may play a significant role in determining its function [12–15]. We observed a high percentage of cases showing p-Akt nuclear positivity in the epithelial compartment in all categories studied, including NM and OLP. Only oral premalignant lesions (OL) demonstrated higher levels of nuclear p-Akt expression compared to OLP. On the other hand, cytoplasmic detection of p-Akt rendered different results: all NM cases were negative, whereas only 5% of OLP cases studied were positive. In contrast, a significant proportion of OL and OSCC cases (about 65% and 70%, resp.) were positive for cytoplasmic p-Akt. These results, taken together, suggest that cytoplasmic p-Akt may be a more meaningful indicator of the biological activity of the molecule in the context of oral carcinogenesis, which remains undetected under normal conditions and seems to be activated in the majority of OL and OSCC cases, as opposed to only a few OLP cases.

In the study of Pontes et al. [10], p-Akt was detected in all cases of NM, OL, and OSCC. Both severe dysplasia and OSCC cases showed higher p-Akt expression compared to NM; in addition, OSCC cases demonstrated higher levels of p-Akt immunostaining compared to dysplasias. On the other hand,

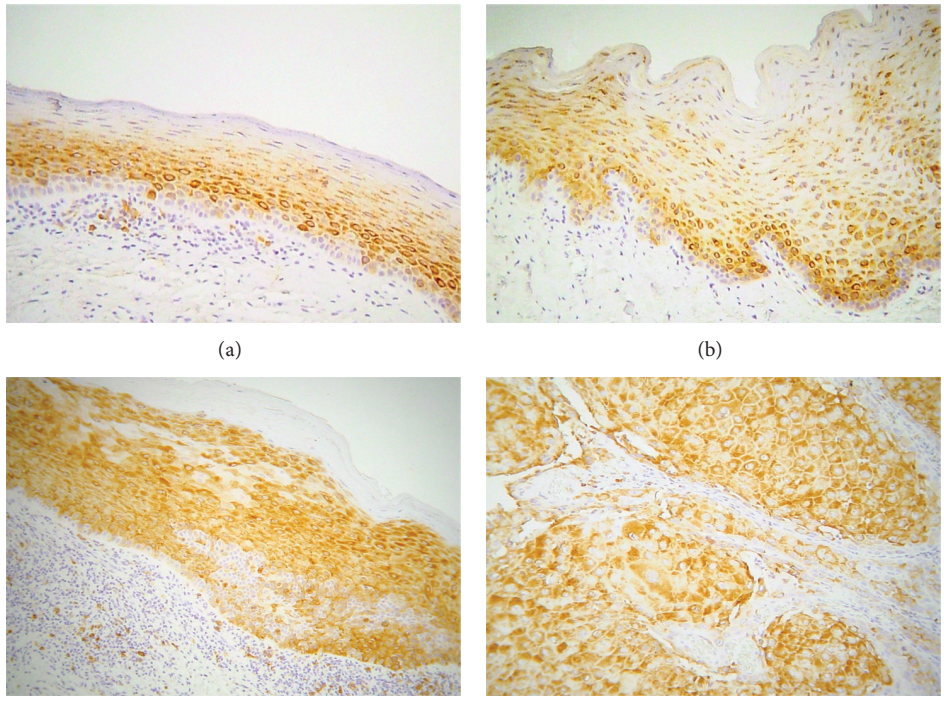
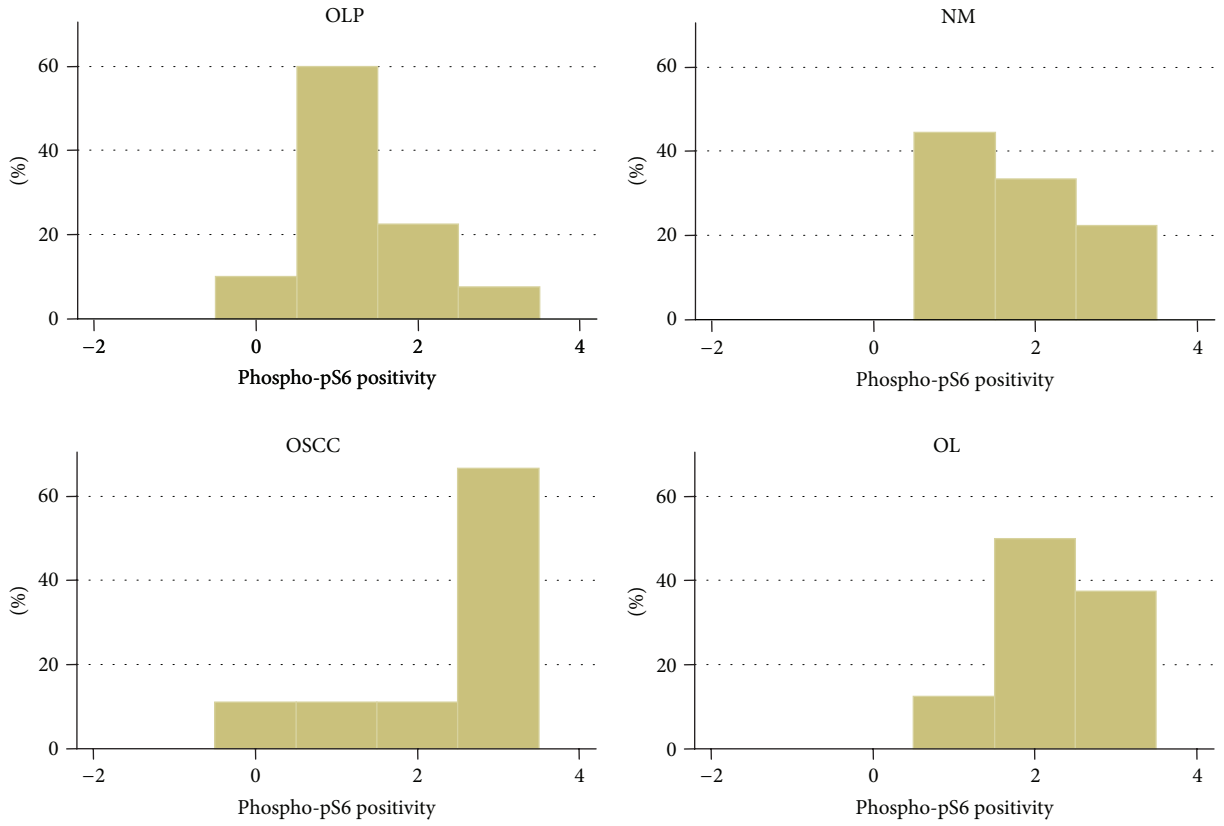


Figure 6: Immunohistochemical expression of phosphorylated ribosomal protein pS6 (phospho-pS6) in selected cases of (a) oral lichen planus (OLP), (b) normal mucosa (NM), (c) oral leukoplakia (OL), and (d) oral squamous cell carcinoma (OSCC) (immunohistochemistry, 100x magnifications).

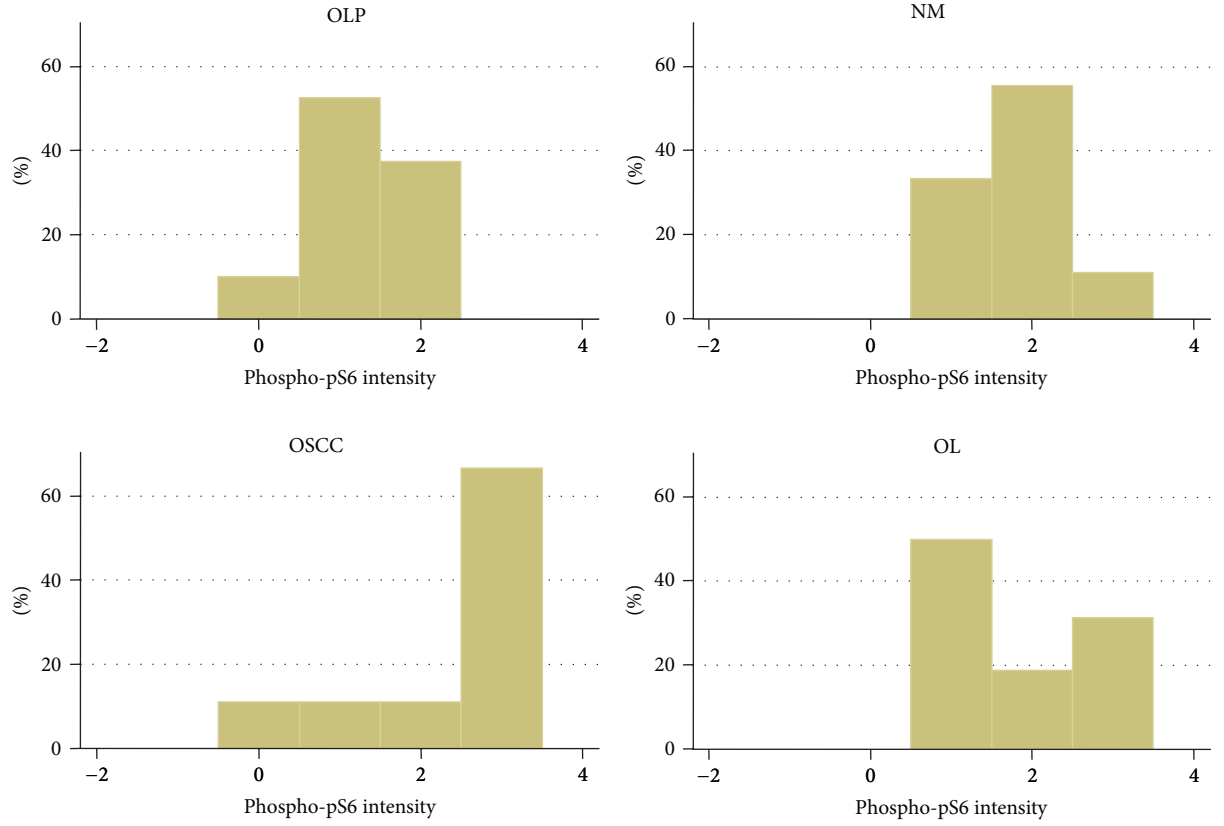
no significant differences were observed among the three histological grades of oral dysplasia. Regarding the localization of immunostaining, Pontes et al. [10] reported that p-Akt was expressed mainly in both cytoplasmic and nuclear compartments, although restricted staining in either the nucleus or the cytoplasm was also occasionally observed. de Freitas Silva et al. [11] also evaluated p-Akt immunohistochemical levels in oral lesions and reported a significant increase in OL and OSCC compared to NM; no significant differences among OL cases with different degrees of dysplasia were noted. With regard to localization, de Freitas Silva et al. [11] reported that NM and OL cases showed p-Akt immunoreactivity limited to the nucleus, whereas OSCC cells expressed both nuclear and cytoplasmic immunostaining. The authors suggested that p-Akt may participate in the multistep process of oral carcinogenesis and may be associated with TWIST expression, a molecule involved in epithelial-mesenchymal transition [11]. It should be noted that the antibody used by Pontes et al. [10] and de Freitas Silva et al. [11] was specific for detecting p-Akt phosphorylation at threonine 308. In addition, analysis of the immunostaining was not performed separately in the nucleus and the cytoplasm of epithelial cells. Similar to our study, Wu et al. [21] analyzed the immunohistochemical expression of p-Akt in NM, OL, and OSCC, but not in OLP, using an antibody against Akt phosphorylated at serine 473. Interestingly, NM showed faint or weak staining, with an occasional lack of expression, which was predominantly located within the nucleus at the basal cell layer. Overall, there was a gradual increase in p-Akt immunostaining from NM to precancerous lesions and OSCCs.

Despite differences in methodology, our findings are in agreement with previous studies in that p-Akt was higher in oral precancerous and cancerous lesions compared to NM. Cytoplasmic p-Akt expression in a minority of OLP cases indicates that this molecule may not participate in the mechanisms underlying OLP pathogenesis. However, it could be hypothesized that certain OLP cases harbor abnormal Akt activity, which could be related to their potential for malignant transformation. In other words, OLP cases with cytoplasmic p-Akt immunostaining may share similar characteristics with OL and OSCC cases showing similar characteristics, thus theoretically being more suspicious for the accumulation of additional genetic and epigenetic alterations leading to cancer development.

One major target of p-Akt is mTOR, which is activated through p-Akt-induced direct phosphorylation and inhibition of TSC2, a tumor suppressor protein that functions as a negative regulator of mTOR [22]. By controlling important downstream targets, mTOR exerts a crucial role in cell fate decisions, so that mTOR signaling dysregulations have been implicated in various forms of human cancer [4, 6, 7]. In this study, p-mTOR was almost exclusively detected in the cytoplasm in 63.2% of OL and 44.4% of OSCC cases, being absent in oral NM. These results indicate that mTOR pathway activation occurs in early stages of oral carcinogenesis. On the contrary, only a minority of OLP cases (10.3%) were

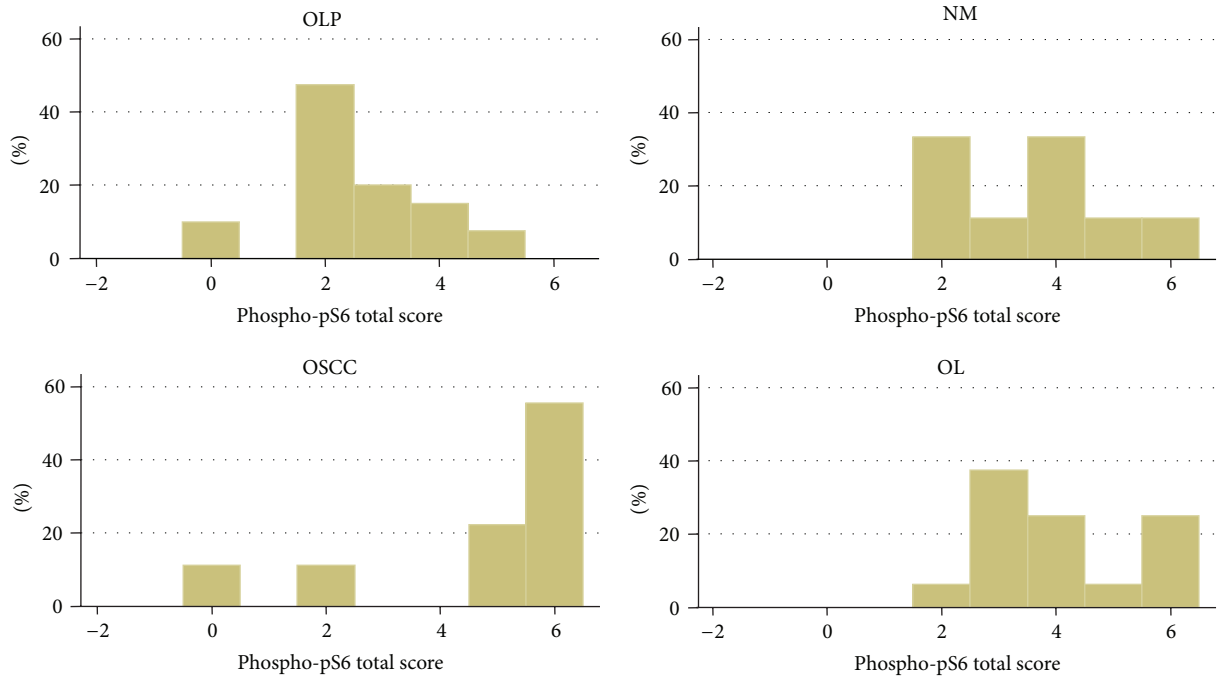


(a)



(b)

Figure 7: Continued.



(c)

Figure 7: Graph of immunohistochemical results for phosphorylated ribosomal protein pS6 (phospho-pS6). Distribution of cases per lesion category according to (a) positivity score, (b) intensity score, and (c) total score. Abbreviations: oLP: Oral lichen planus; NM: normal mucosa; OSCC: oral squamous cell carcinoma; OL: oral leukoplakia.

positive for p-mTOR. In a previous study by Clark et al. [8], p-mTOR immunohistochemical expression was 81.9% sensitive and 100% specific in differentiating head and neck SCC from noncancerous oral mucosa, with the latter being uniformly negative. It is tempting thus to speculate that p-mTOR-positive OLP cases may have an increased potential for malignant transformation compared to p-mTOR-negative cases. If this hypothesis holds true, p-mTOR detection (which may be at least partially associated with cytoplasmic p-Akt positivity) could serve as a marker of increased risk of malignant progression in OLP.

Ribosomal protein S6 (pS6) is one major downstream target and effector of the mTOR pathway. Following activation by the ribosomal protein S6 kinase, phosphorylated pS6 participates in the regulation of cell proliferation, cell growth, and protein synthesis [23]. High expression of activated pS6 has been detected in a number of human cancers suggesting its possible usefulness as a cancer biomarker [12, 24]. Similarly, oral, head, and neck squamous cell carcinoma has demonstrated relatively high phosphorylated pS6 levels [8, 25]. Amornphimoltham et al. [25] have shown that phosphorylated pS6 is expressed at low levels in normal oral mucosa compared to oral dysplasia and SCC, supporting the notion that pS6 activation may represent an early event in the oral carcinogenesis process. In a recent study, Chaisuparat et al. [24] investigated the phosphorylation levels of ribosomal protein S6 in normal oral mucosa, oral epithelial dysplasia, and OSCC cases. Similar to our study, phosphorylated pS6 was detected in the majority of cases studied (including 50% of control normal mucosa samples, 100% of oral epithelial dysplasias, and 88.68% of OSCC). Oral dysplasias and OSCC showed a higher frequency of pS6 phosphorylation compared to normal mucosa. These authors also concluded that pS6 activation represents an early event in oral carcinogenesis and may serve as a useful diagnostic biomarker.

In agreement with the aforementioned studies, our results confirm high activation levels of pS6 in oral precancerous and cancerous lesions. On the other hand, the vast majority of OLP cases showed phospho-pS6 positivity, albeit at significantly lower positivity and intensity levels compared to OL and OSCC; in fact, phospho-pS6 levels did not differ significantly between OLP and NM. Considering that pS6 phosphorylation is mediated by activated mTOR, which, in turn, is to a large extent controlled by Akt activation, our results collectively implicate that Akt/mTOR/pS6 pathway is active in the majority of oral premalignant and malignant lesions. In contrast, OLP cases, as a group, do not seem to be characterized by aberrant activation of the oncogenic Akt/mTOR/pS6 pathway. Nonetheless, the demonstrated variability at phosphorylated protein levels among OLP cases may indicate that a minority of OLPs do possess exuberant Akt/mTOR/pS6 signaling activity (manifested by positivity for p-Akt and p-mTOR and higher levels of phosphor-pS6), which could contribute to their premalignant potential.

It should be emphasized that this is the first attempt to assess the actual activation status and role of Akt/mTOR/pS6 signaling in OLP. Using a bioinformatics model based on the "leader gene approach," Giacomelli et al. [26] have suggested

that PI3K signaling events mediated by Akt may play a role interrelating OLP and OSCC.

In conclusion, the present study revealed that activation of the Akt/mTOR/pS6 signaling is a common event in oral premalignant (OL) and malignant (OSCC) lesions but occurs only in a subset of OLP cases. It is interesting to note that differences in the localization of p-Akt in the nuclear and cytoplasmic compartments may account for a variable activation status, a notion that needs further investigation. In addition, investigating the expression and activation levels of other molecules involved in the complex mTOR signaling may provide more information on the actual activation status of this pathway in oral carcinogenesis. Although no follow-up data were available to record the malignant transformation rate of OLP cases studied, it is conceivable that lesions harboring aberrations in the Akt/mTOR/pS6 signaling may bear a closer molecular similarity to actual premalignant lesions and may be at increased risk for cancer development. Furthermore, the advent of molecular targeted therapies against Akt and mTOR holds promise for their use in the context of premalignant disease, which may encompass selected cases of OLP. Confirmation of these hypotheses will necessitate a large prospective study of OLP cases with careful recording of the clinical features and long follow-up. It will be interesting to correlate the presence and frequency of molecular aberrations involving the Akt/mTOR/pS6 or other related pathways with specific OLP clinical subtypes, given that specific clinical forms of the disease (such as erosive, atrophic, and/or plaque-like OLP) have been associated with a higher malignant transformation rate [1, 2]. Moreover, long-term follow-up will reveal those cases demonstrating malignant transformation (or even the development of a preceding stage of histologic features of dysplasia) allowing the determination of the actual prognostic significance and clinical value of specific molecular markers.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Verrucous Papillary Lesions: Dilemmas in Diagnosis and Terminology

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Verrucous papillary lesions (VPLs) of oral cavity are diagnostically challenging as they include a spectrum of benign, potentially malignant, and frankly malignant lesions. A majority of the benign VPLs have viral aetiology and include commonly occurring squamous papilloma along with verruca vulgaris, focal epithelial hyperplasia, and condyloma. Current understanding of potentially malignant VPLs is perplexing and is primarily attributed to the use of confusing and unsatisfactory terminology. Clinically and histologically oral verrucous hyperplasia, a potentially malignant disorder, resembles oral verrucous carcinoma and may be indistinguishable from one another. The most reliable way to separate these entities on routine haematoxylin-eosin stained tissue sections is to recognize the exophytic growth patterns of oral verrucous hyperplasia from the combined exophytic and endophytic growth patterns associated with verrucous carcinoma. A review of the literature showed that there is a lot of confusion regarding the current clinical and histopathological guidelines to diagnose this potentially malignant entity. The criteria elaborated by different authors in establishing the diagnosis of oral verrucous hyperplasia are discussed in detail. A brief overview of the treatment modalities adopted is also discussed. The need for establishing a clear understanding of this potentially malignant entity is stressed as it may have far reaching implications on its management.

1. Introduction

Verrucous papillary lesions (VPLs) of the oral cavity are diagnostically challenging as they include a spectrum of benign, potentially malignant, and frankly malignant lesions. A majority of the benign VPLs have a viral aetiology and include the more commonly occurring squamous papilloma along with verruca vulgaris, focal epithelial hyperplasia, and condyloma [1]. Mucosal HPV types (HPV 6, 11, 13, 30, 32, 45, 52, 55, 59, 69, 72 and 73) have been isolated from these oral lesions [2]. Histopathologically, these benign lesions do not demonstrate any cellular atypia. It is sometimes difficult to distinguish these lesions, but clinical and certain histological features facilitate their diagnosis [3, 4].

Benign VPLs with known aetiologic factors will not be the focus of our discussion in this paper. Our current understanding of potentially malignant VPLs is perplexing and is primarily attributed to the use of confusing and unsatisfactory terminology. This may be best exemplified by verrucous hyperplasia, a potentially malignant disorder presenting as a verrucous or exophytic growth characterized by keratosis and/or varying grades of dysplasia [1]. Verrucous hyperplasia is a histopathological entity with clinical features that may be indistinguishable from a verrucous carcinoma [5]. The pathologist may fail to convey to the clinician the potentially malignant nature of verrucous hyperplasia due to the absence of overt features of dysplasia, and the clinician may subsequently consider this as a benign condition. This may become further established when reactive lesions such as inflammatory papillary hyperplasia may also be casually diagnosed as verrucous hyperplasia both clinically and histopathologically.

2. Historical Background and Terminologies

The first ever documented evidence of a VPL dates back to 1941 when Fridell and Rosenthal reported a case of well-differentiated squamous cell carcinoma of the oral cavity as "papillary verrucoid carcinoma." In 1948, Ackerman reported a series of thirty one similar cases and coined the term "verrucous carcinoma" [6]. Ackerman is credited with the recognition of distinctive clinical and microscopic features of verrucous carcinoma that he considered to be a variant of squamous cell carcinoma. A relatively high proportion of these lesions tend to involve the buccal mucosa in tobacco chewers [7]. These lesions are markedly exophytic and endophytic with a tendency to erode the underlying tissues including bone. Histomorphologic features include densely parakeratinized papillary surface, deep clefts in the epithelium, blunt and voluminous rete ridges with little or no dysplastic changes exhibiting a pushing border effect, and an intact basement membrane [8]. Ever since its original description within the oral cavity, there have been reports of similar lesions occurring at other sites including the larynx, perianal region, cervix, and glans penis [6]. Many of these later reports have remained true to the original description by Ackerman; however, challenges exist in recognizing an optimal therapeutic approach, the incidence of recurrence, and frequency of anaplastic transformation of verrucous carcinoma [9]. The general guidelines for the management of verrucous carcinoma of the head and neck recognize surgical excision as the primary treatment although the opinion is divided among investigators on the role of radiotherapy alone or as an adjunct to surgery as a treatment modality. The primary cause of concern is reflected in the views that irradiation of verrucous carcinoma is less effective and more likely to result in a recurrence with a more aggressive cancer through anaplastic differentiation [10]. Carcinoma cuniculatum is a variant of verrucous carcinoma and has also been described under an array of confusing terminologies including inverted verrucous carcinoma and oral florid papillomatosis to name a few [11].

Florid oral papillomatosis (FOP) is a rare disorder of the oral cavity and lips, characterised by the presence of multiple and multifocal papillomatous and verruciform growths that form confluent plaques and vegetation. It was originally recognized by Rock and Fisher [12] to describe multiple papillary lesions involving the mouth and larynx [13]. Malignant transformation has been reported in a subset of these cases. Strangely enough different, terminologies such as verrucous hyperplasia, verrucous leukoplakia, and papillomatosis mucosae carcinoides have been used synonymously to describe this lesion [14]. The histopathologic feature of FOP consisting of papillomatous and acanthotic as well as partially keratinized epithelium with elongated rete ridges is distinct from a verrucous carcinoma [14]. However, in the older literature, this lesion is considered to be synonymous with verrucous carcinoma [8].

Ackerman and McGavran [15] introduced the term "verrucous hyperplasia" to describe a condition that closely resembles verrucous carcinoma clinically and histologically. A subsequent review by Adkins and Monsour [16] concluded that an entity described as verrucous leukoplakia by many authors may actually correspond to some forms of verrucous hyperplasia [5]. Shear and Pindborg were the first to perform a detailed clinical and histological analysis of verrucous hyperplasias of the oral mucosa and unified previously used terms such as verrucous leukoplakia under this distinct histologic subset within the leukoplakia family of clinically identified lesions [17]. Clinically verrucous hyperplasias have been classified into two variants, a sharp variety (Figure 1) comprising long, narrow, and heavily keratinized verrucous processes which appears white as result of heavy keratinization. This entity may represent the form referred to as verrucous leukoplakia by many authors. The second clinical variant is a blunt variety (Figures 2 and 3) consisting of verrucous processes that are broader, flatter, and not heavily keratinized. In a majority of cases, areas of homogeneous leukoplakia are an integral component of the lesion and of the mucosa elsewhere in the mouths of the same patients [5]. Histologically, epithelial dysplasia is a prominent feature in verrucous hyperplasias and these lesions have been found to be juxtaposed with verrucous carcinoma and squamous cell carcinoma in a significant percentage of patients [17]. We propose that all lesions whether they are of the blunt/sharp-type should be relabelled as oral verrucous leukoplakia clinically. However, confusion persists with regard to the blunt-type lesions which are red, and it may not be possible to categorize them as verrucous leukoplakia. It is recommended that all these lesions should be diagnosed histologically as verrucous hyperplasia based on the following criteria subject to a consensus. The proposed histopathologic criteria for diagnosis of oral verrucous hyperplasia are as follows:

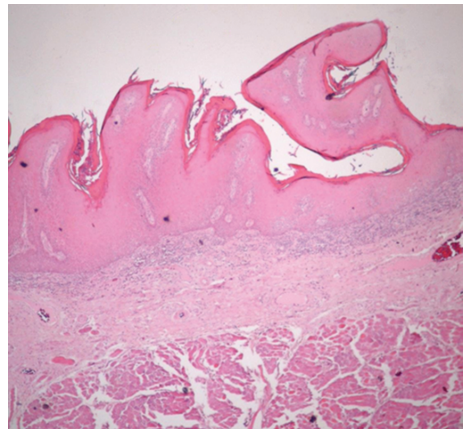

Figure 1: Photomicrograph of verrucous hyperplasia showing sharp surface projections (original magnification 4x, H and E stain).

(a) long and narrow heavily keratinized verrucous processes or broad and flat verrucous processes that are less keratinized;

(b) absence of invasion of the hyperplastic epithelium into the lamina propria as compared with the adjacent normal mucosal epithelium;

(c) presence of cytologic/architectural features of dysplasia.

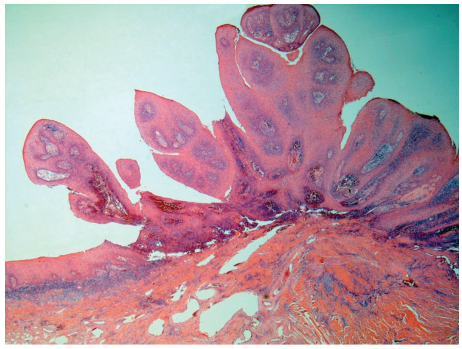

Figure 2: Photomicrograph of verrucous hyperplasia showing blunt surface projections (original magnification 4x, H and E stain).

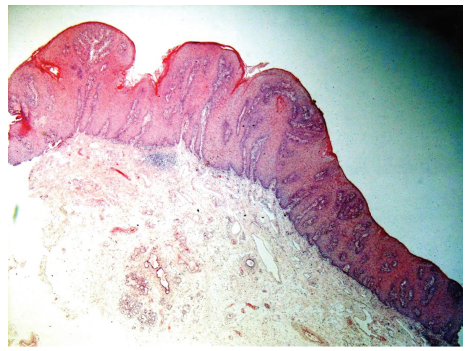

Figure 3: Photomicrograph of verrucous hyperplasia showing blunt surface projections (original magnification 4x, H and E stain).

Oral verruciform leukoplakia is not firmly established in the literature. This terminology was proposed by Wang et al. to denote a subset of oral verrucous hyperplasias that strongly resemble verrucous leukoplakia clinically [18].

3. Diagnostic Dilemmas

3.1. Clinical Spectrum of Oral Verrucous Hyperplasia. It has been observed that leukoplakias may evolve through verrucous hyperplasias, verrucous carcinomas, and eventually squamous cell carcinomas [5]. A similar observation by Slootweg and Muller led them to hypothesize that verrucous hyperplasias and verrucous carcinomas represent a spectrum of the same process which represents a ubiquitous premalignant change in the whole oral mucous membrane [19]. The characteristic histologic feature that distinguishes a verrucous carcinoma is the presence of microscopic verrucous projections and endophytic epithelial extensions into the underlying lamina propria of which the latter are conspicuously absent in verrucous hyperplasia [17]. Hansen et al. [20] in his long term study on proliferative verrucous leukoplakia (PVL) considered verrucous hyperplasia and verrucous carcinoma as intermediate clinicopathological stages in its spectrum. This was subsequently confirmed by Batsakis et al. [21]. Originally described by Hansen et al. [20], PVL is a recognized specific type of nonhomogeneous leukoplakia with an extremely high propensity for malignant transformation [22]. Verrucous carcinomas exist within the histologic continuum ranging from benign squamous hyperplastic lesions and proliferative verrucous lesions to invasive squamous cell carcinoma. Distinguishing verrucous carcinoma from these similar benign and malignant processes may be difficult. The belief by earlier researchers that verrucous carcinomas may evolve into a conventional invasive squamous cell carcinoma may be due to presence of small foci of squamous cell carcinoma in those lesions with dominant features of verrucous carcinoma. Some investigators consider these verrucous squamous carcinomas to be "hybrid" forms of verrucous carcinoma or a squamous cell carcinoma with verrucoid features [10]. We firmly believe that addition of new terminologies may add to further confusion, and therefore, the two entities—verrucous carcinoma and squamous cell carcinoma—should remain independent. It is strongly recommended that surgical specimens of verrucous carcinoma should be thoroughly sampled to avoid missing an occult focus of conventional squamous cell carcinoma. This will provide valuable information on management as these two lesions have a different prognosis.

3.2. Clinical Variants of Oral Verrucous Hyperplasia. Wang et al. [18] discussed in detail the clinicopathologic features and behaviour of verrucous hyperplasia in sixty Taiwanese patients. Contrary to the earlier accepted histological subtypes as originally proposed by Shear and Pindborg, Wang reclassified these lesions into (1) plaque-type and (2) mass-type based on their histological features. The histologic criteria for diagnosis were primarily epithelial hyperplasia with parakeratosis or hyperkeratosis and a verrucous surface. An absence of invasion of the hyperplastic epithelium into the lamina propria as compared with the adjacent normal mucosal epithelium was an additional important histologic criterion for diagnosis. A surface keratin layer of $>40\,\mu$ thickness was accepted as a differentiator between the two subtypes. Lesions exhibiting a verrucous surface with single or multiple protruding masses of epithelial growth showing minimal connective tissue cores and a surface keratin thickness of $<40\,\mu$ were designated as the mass type. The plaque-type lesions demonstrated a verrucous surface, epithelial hyperplasia, and a surface keratin thickness of $>40\,\mu$. Clinically, the mass-type verrucous hyperplasia manifested as single or multiple mass-like verrucous whitish pink lesions while the plaque-type lesions appeared as whitish verrucous plaques. Dysplasia was not a determinant in the diagnosis of these lesions. However, in this study, the plaque-type lesions exhibited a greater frequency of epithelial dysplasia [18].

Wang et al. remarked that they had difficulty in correlating the clinical and histopathological features of the plaque-type lesions. The histopathological features of these lesions conferred with their criteria but, a subsequent clinical reevaluation led them to reclassify these lesions as oral verruciform leukoplakia. Thus, they concluded that the terminology oral verrucous hyperplasia should be used to denote the mass-type lesions both clinically and histologically. They also suggested that the plaque-type lesions should be clinically classified as oral verruciform leukoplakia and histologically as verruciform hyperplasia [18].

Our literature review from Taiwan also confirms that there is a general consensus among the various authors there regarding the clinical and histological characteristics of oral verrucous hyperplasia which is in agreement with guidelines proposed by Wang et al. as described earlier [23, 24]. There does not seem to be any differences of opinion regarding the potentially malignant nature of oral verrucous hyperplasias and its association with high risk habits such as tobacco and areca quid chewing and cigarette smoking. The primary sites of involvement include the buccal mucosa, vestibular mucosa, gingiva, and alveolar mucosa which are a reflection of a direct cause and effect relationship associated with risk habits [5, 18, 25, 26]. Clinically, they are manifested as white to whitish pink lesions that can be attributed to variations in the degree of keratinization.

The clinical appearance of these lesions has not generally been well characterised as much emphasis has been laid on the verrucous/exophytic nature of these lesions with little attention being given to colour variation. Moreover, these lesions are considered to be clinically indistinguishable from verrucous carcinomas which are generally white or greyish white in colour [8, 27, 28]. A significant association with leukoplakia has been stressed in the literature. These areas of homogeneous leukoplakia occur adjacent to oral verrucous hyperplasia and histologically represent hyperkeratosis and epithelial dysplasia. This as well as evidence from long term follow up of patients with oral leukoplakia suggests that oral verrucous hyperplasia actually represents a process in continuum [5, 29]. Recognition of this feature is subdued in many of the reports from Taiwan where the lesions are primarily described as either the mass or the plaque types. A combination type lesion with a peripheral plaque and a central mass reported in the Taiwanese literature may be indicative of the associated leukoplakia [18, 24]. This needs to be addressed as reports from Taiwan may represent a subset of verrucous hyperplasia where changes in the adjacent mucosa are less pronounced. This has to be approached with caution bearing in mind the concept of field cancerization where abnormal, hyperplastic, and often atypical epithelium clinically visible as leukoplakia or atrophic epithelium may represent an area that has been preconditioned by a carcinogen to develop into a malignancy [30].

4. Histopathological Features of Oral Verrucous Hyperplasia

Histologically, oral verrucous hyperplasia resembles oral verrucous carcinoma and may be indistinguishable from one another. The most reliable way to separate these entities on routine haematoxylin-eosin stained tissue sections is to recognize the exophytic growth pattern of oral verrucous hyperplasia from the combined exophytic and endophytic growth pattern associated with a verrucous carcinoma. In oral verrucous carcinoma, the projections of neoplastic epithelium are seen deep to the adjacent uninvolved epithelium, whereas, in oral verrucous hyperplasia, they are seen only at the same level as the adjacent epithelium. However, separation of these lesions is often obscured by small biopsies, poorly orientated specimens, and biopsies that fail to demonstrate the lesion margins [27]. The aforementioned histological features of oral verrucous hyperplasia are unanimously recognized as a prerequisite for its diagnosis, whereas dysplasia is not. Epithelial dysplasia with a propensity for moderate dysplasia has been reported in a majority of cases of verrucous hyperplasia ranging from 18 to 68% [5, 18, 26]. In a hospital based follow-up study from Taiwan where oral verrucous hyperplasia is very common, the malignant transformation rate was estimated at 20% in a cohort of forty-four male subjects with verrucous hyperplasia. This was only second to epithelial dysplasia which exhibited the highest rate of malignant transformation of 24% [25]. These estimates may further add to the confusion as it has not been clearly stated whether cases classified as epithelial dysplasia also included cases of verrucous hyperplasia with dysplasia in the first place.

5. Treatment of Oral Verrucous Hyperplasia

Poswillo is of the opinion that oral verrucous hyperplasia and verrucous carcinoma should be managed similarly because of the significant overlap in their clinicopathologic features [31]. Many reports consider oral verrucous hyperplasia as a potentially malignant disorder [18, 25]. However, it has not been listed so by the WHO [32]. It is well established that verrucous carcinoma is a low grade malignancy. It is also clear that verrucous hyperplasia is a forerunner of verrucous carcinoma, and transition to the latter is quite consistent. Hence there is an opinion that the two lesions should be managed identically [33]. Verrucous carcinoma has been treated with different modalities such as excision with or without radical surgery, chemotherapy, radiation, or a combination of these modalities [29]. Surgery is the most common treatment modality, while the use of radiotherapy is controversial. The conventional treatment of oral verrucous hyperplasia has been total surgical excision. Recurrence and/or transformation of oral verrucous hyperplasia to either verrucous carcinoma or conventional SCC have been reported after surgical intervention. Shear and Pindborg reported recurrence in four of their patients with lesions showing both verrucous hyperplasia and verrucous carcinoma [5]. This may be subject to the use of strict criteria for defining recurrence and differentiating it from residual lesions. Wide surgical excision of the primary verrucous lesion with adequate mucosal and soft-tissue margin is necessary to avoid local recurrence [33].

6. Conclusion

It is evident from our discussion that clearer guidelines for recognizing the clinical and histopathological features should be established to diagnose oral verrucous hyperplasia. It is proposed that both the sharp and the blunt varieties of oral verrucous hyperplasia as originally recognized by Shear and Pindborg and subsequently relabelled as the plaque-type and mass-type lesions should be best diagnosed clinically as a non-homogeneous leukoplakia or more specifically as verrucous leukoplakia [32]. Following histological evaluation, the lesions may be further characterised as oral verrucous hyperplasia subject to a consensus.

Recommendation

In this context, we recommend a consensus workshop to determine an acceptable clinicopathological guideline for recognizing oral verrucous hyperplasia. This will eliminate the use of conflicting terminologies in the future that would otherwise plague an overburdened vocabulary for characterizing these lesions.

Disclosure

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Conflict of Interests

The authors have no conflict of interest to declare in relation to the work presented in this paper.

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Review Article

Advances in Optical Adjunctive Aids for Visualisation and Detection of Oral Malignant and Potentially Malignant Lesions

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Traditional methods of screening for oral potentially malignant disorders and oral malignancies involve a conventional oral examination with digital palpation. Evidence indicates that conventional examination is a poor discriminator of oral mucosal lesions. A number of optical aids have been developed to assist the clinician to detect oral mucosal abnormalities and to differentiate benign lesions from sinister pathology. This paper discusses advances in optical technologies designed for the detection of oral mucosal abnormalities. The literature regarding such devices, VELscope and Identafi, is critically analysed, and the novel use of Narrow Band Imaging within the oral cavity is also discussed. Optical aids are effective in assisting with the detection of oral mucosal abnormalities; however, further research is required to evaluate the usefulness of these devices in differentiating benign lesions from potentially malignant and malignant lesions.

1. Introduction

Oral cancer affects the lips, tongue, gingiva, floor of mouth, palate, tonsils, and oropharynx [1–3]. It is ranked the sixth most common malignancy worldwide and is diagnosed at an increasing rate [4], with an estimated 263,900 new cases and 128,000 deaths in 2008 alone [5]. Oral squamous cell carcinoma (OSCC) can affect any tissue lined with oral mucosal epithelium and accounts for 90% of oral malignancies [1, 4]. Known aetiological risk factors for OSCC include tobacco, betel quid, alcohol, and micronutrient deficiency [2, 6, 7]; however, recent studies also implicate human papillomavirus (HPV) as a causative factor in cancers of the base of the tongue, tonsils, and oropharynx in patients without traditional risk factors [1, 2, 7].

Despite advances in cancer therapies, the five-year survival rate for oral cancer has remained at approximately 50% over the past three decades [4, 8]. This is primarily due to delayed diagnosis, with approximately half of all oral cancers diagnosed at stages III or IV [9]. By these stages, lymphatic spread has occurred and treatment is for a systemic condition rather than a localized disease process. Localised cancers have survival rates of up to 83% but this falls to 32% once tumour metastasis has occurred [10]. As such, emphasis should be placed on earlier detection of oral cancers to improve patient survival rates.

OSCC is often preceded by visible and histological changes in the oral mucosa. Conditions which have the potential to develop into malignancies are referred to as oral potentially malignant disorders (OPMDs) and these include leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen planus, and actinic keratosis [11]. Although only a small proportion of OPMDs undergo malignant transformation, the key to improved patient prognosis is believed to be through early detection and management of these lesions [12, 13].

The current protocol for detecting OPMDs by conventional oral examination (COE) involves visual inspection of the oral cavity and tactile examination of head and neck lymph nodes by a medical or dental practitioner. However, even with meticulous follow up, early malignant changes are still overlooked using COE [14] as dysplasia may be found in clinically normal mucosa [15, 16]. While a recent meta-analysis reported 93% sensitivity for COE, specificity was poor at only 31%. Therefore, COE cannot reliably differentiate between benign and dysplastic lesions, and this is most likely due to the fact that a number of benign conditions mimic oral

malignancies [15]. Epstein et al. [15] suggested that further research into adjunct visualisation technologies is required to improve the reliability of clinicians in screening for malignant and potentially malignant disorders.

A variety of devices utilising the principles of tissue autofluorescence, tissue reflectance, or narrow band imaging (NBI) have been commercialised as adjunctive aids to COE for the detection of OPMDs and OSCCs. This paper critically appraises the literature regarding commercially available devices VELscope, Identafi, and Narrow Band Imaging, and discusses their application in the oral cavity, as well as highlighting other approaches to optical imaging.

2. VELscope

Autofluorescence is a phenomenon whereby an extrinsic light source is used to excite endogenous fluorophores such as certain amino acids, metabolic products, and structural proteins [26]. Within the oral mucosa, the most relevant fluorophores are nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) in the epithelium and collagen cross-links in the stroma [27]. The fluorophores absorb photons from the exogenous light source and emit lower energy photons which present clinically as fluorescence [28]. Each fluorophore is associated with specific excitation and emission wavelengths.

Mucosal abnormalities can alter the absorption and scattering properties of tissue due to changes in tissue architecture and concentrations of fluorophores. *In vitro* studies have shown a decrease in autofluorescence in oral epithelial dysplasia (OED) as well as mucosal inflammation [27, 29]. Multiple oncological applications for *in vivo* fluorescence spectroscopy have previously been described [30]. Preliminary research indicates that autofluorescence is a suitable adjunct to COE in early detection of OSCC and OPMD [28, 31–34].

VELscope (LED Medical Diagnostics Inc., Barnaby Canada) utilises blue light excitation between 400 and 460 nm wavelength [1, 35–37] to enhance oral mucosal abnormalities by direct tissue autofluorescence. At these excitation wavelengths, normal oral mucosa is associated with a pale green fluorescence when viewed through a filter, whereas abnormal tissue is associated with a loss of autofluorescence (LAF) and appears dark [1]. Although pilot studies found that these excitation wavelengths could be used *in vivo* to differentiate normal oral mucosa from dysplasia, carcinoma in situ (CIS), and invasive carcinoma, the manufacturer extrapolated these findings to indicate that VELscope can help detect oral mucosal abnormalities not visible under white light examination [33, 38].

Early research supporting the use of VELscope is comprised of case reports regarding its use on referred or review patients at specialist oral dysplasia clinics [17, 39]. Kois and Truelove [39] found that VELscope assisted in the detection of dysplastic and malignant lesions not visible by COE and helped raise suspicion of lesions which would otherwise not be subjected to biopsy. In one particular case where widespread erythema was present, VELscope revealed an area which later proved to be a well-differentiated carcinoma. It also demonstrated its value in demarcating margins of established tumours where the malignant tissue extended beyond what was otherwise clinically visible [25]. While the use of VELscope was in specialist environments, these case reports provided initial evidence that the device enabled clinicians to differentiate dysplasia from normal oral mucosa.

The diagnostic accuracy of VELscope in detecting dysplasia and OSCC has been studied extensively in specialist referral centres [18–20, 22, 24, 40], with reported sensitivities ranging from 30 to 100% (Table 1) [18–20, 22, 24, 40]. Despite the large range, some studies noted that VELscope helped discover dysplastic lesions missed by COE [19, 40]. For these reasons, VELscope appears to be a valuable tool in monitoring patients with a history of head and neck cancer. However, Mehrotra et al. [22] argued that since not all dysplastic lesions displayed LAF, its use in routine practice should be discouraged as it can result in missed lesions and a false sense of security. Of concern, VELscope has a fairly high rate of false positives, with reported specificities ranging between 15 to 81% (Table 1) [18–20, 22, 24, 40]. This suggests that VELscope is a poor differentiator between benign and dysplastic lesions [18, 20]. In particular, inflammatory lesions typically display LAF as well and, thus, act as confounders when using VELscope [19]. As the majority of oral mucosal lesions seen in general practice are benign in nature, incorrect interpretation can lead to overestimation of oral mucosal abnormalities and patient harm through unnecessary referrals and biopsies.

While the majority of studies have evaluated VELscope's diagnostic capabilities and accuracy without taking into account clinical characteristics, VELscope's main function is to serve as an adjunctive aid to rather than a replacement for COE. For this reason, Farah et al. [19] prospectively evaluated the use of VELscope in conjunction with COE in a specialist environment. Lesions which displayed diascopic fluorescence were considered negative for LAF. Sensitivity was higher when VELscope and COE findings were combined than for either COE or VELscope alone, whereas specificity only increased slightly [19]. This highlights the importance of clinical interpretation when using VELscope rather than relying on LAF on its own. The authors also assessed the effect of diascopic fluorescence, whereby lesions displaying LAF return to a normal fluorescence pattern with the application of pressure (Figure 1). This technique enables clinicians to differentiate inflammatory lesions from neoplasia since inflammatory lesions typically display complete diascopic fluorescence, whereas neoplastic lesions do not [19]. These results cannot be generalised to general practitioners as the study was performed by specialists, and advanced knowledge of mucosal pathology is beneficial to effectively differentiate between LAF and diminished autofluorescence [19, 20, 24]. Furthermore, Farah et al. [19] observed that complete blanching of lesions was difficult to achieve and partial blanching could complicate interpretation. Therefore, VELscope is vulnerable to interoperator variability [24].

Thus far, research on the use of VELscope for routine screening in the general population is limited (Table 2). Huff et al. [41] found an increased rate of detection of OED using VELscope in a private practice setting when compared to

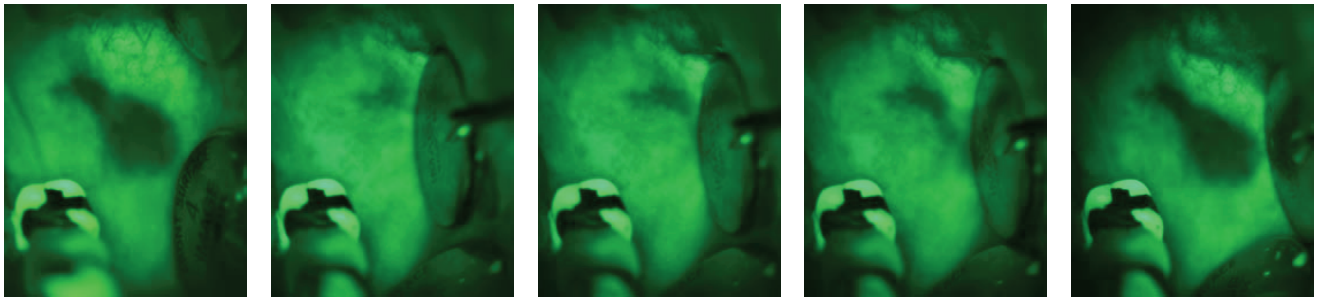
Table 1: Published papers on the use of VELscope in specialist practice.

Purpose	Paper	Type of study	Sample population	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Notes
Used to detect OPMDs only	Poh et al. [17]	Case report	3 case reports of patients with a history of either oral dysplasia or CIS.	—	—	—	—	—	All cases demonstrated LAF in the area where there was a lesion. 1 patient had moderate epithelial dysplasia, another had CIS, and the third case had both severe dysplasia and CIS.
	Awan et al. [18]	Prospective cohort study	126 patients with oral white or red lesions suspicious of OPMD.	84	15	38	61	—	Of the 126 lesions, 7 dysplasias had no LAF and 61 nondysplastic lesions had LAF.
	Farah et al. [19]	Prospective cohort study	112 patients with white or mixed red-white lesions suspicious of OPMD.	30	63	19	75	55	Interpretation based on VELscope findings only.
				46	68	29	82	63	Interpretation based on both COE and VELscope findings.
	Rana et al. [20]	Cross-sectional study	289 patients in total with an OPMD (166 patients examined with COE, 123 patients examined with both COE and VELscope).	100	74	—	—	—	VELscope had higher sensitivity (100% versus 17%) but lower specificity (74% versus 97%) when compared to COE.
Used to detect OPMDs and/or oral cancer	Lane et al. [21]	Prospective cohort study	44 patients with biopsy-confirmed oral dysplasia or OSCC.	98	100	100	86	—	91% of severe dysplasia and CIS showed LAF. 100% of OSCCs had LAF.
	Mehrotra et al. [22]	Cross-sectional study	258 patients in total with clinically innocuous lesions (102 patients were examined with ViziLite; 156 patients were examined with VELscope).	50	39	6	90	—	6 dysplasias did not display LAF. VELscope did not detect any additional lesions following COE.
	Koch et al. [23]	Prospective cohort study	78 patients with clinically diagnosed SCC or suspicious epithelial lesion.	97	96	94	98	—	For diagnosing SCC only.
				94	98	97	96	—	For diagnosing SCC/dysplasia.
	Scheer et al. [24]	Prospective cohort study	64 patients referred to specialist clinic to rule out OSCC.	100	81	55	100	—	False-positive rate of 15.6%. LAF significantly associated with dysplasia or invasive carcinoma when compared to normal tissue ($P < 0.0001$).

Table 1: Continued.

Purpose	Paper	Type of study	Sample population	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Notes
Used to detect oral cancer only	Poh et al. [25]	Prospective cohort study	20 consecutive patients with biopsy-confirmed oral cancer.	—	—	—	—	—	All tumours showed LAF, with a significant correlation between high-grade dysplasia and LAF ($P < 0.0001$). 95% (19/20) of tumours had VELscope margins that extended beyond the clinically visible tumour.

OPMD: oral potentially malignant disorder; CIS: carcinoma in situ; LAF: loss of autofluorescence; COE: conventional oral examination; OSCC: oral squamous cell carcinoma.



(a) (b) (c) (d) (e)

Figure 1: Oral lichen planus on left buccal mucosa displaying loss of autofluorescence when visualised using VELscope (a). The same lesion displaying diascopic fluorescence on application of pressure (b)–(d), returning to its original appearance when pressure is removed (e).

COE. However, this was conducted in parallel cohorts and the clinical characteristics of lesions discovered with VELscope were not discussed. It is unclear whether VELscope aided the detection of new lesions or helped raise the suspicion of lesions detected by COE. Nonetheless, similar results were found during routine screening of patients attending student clinics at a dental school [42]. McNamara et al. [43], however, found the low specificity of VELscope to be a barrier for its use in routine screening in general practice, arguing that it would lead to a large number of overreferrals. In their protocol, the authors did not consider VELscope findings in context by reexamining areas with LAF clinically, despite the fact that LAF alone has little meaning without assessing the site again by COE to eliminate contribution of inflammatory, pigmented, or vascular lesions to this phenomenon. In addition, a case of moderate dysplasia of the lip did not display LAF, repeating concerns that dysplastic lesions may be missed. Future studies assessing the efficacy of VELscope in routine practice should consider both clinical and VELscope findings to assess how these collectively impact on the specificity of the device. Consideration of a tested algorithm and decision making protocol would be helpful for general practitioners utilising the device.

The existing literature indicates that VELscope can differentiate between normal mucosa and mucosal abnormalities; however, it is not highly specific in detecting OPMDs and as a result gives rise to a high rate of false positives. The sensitivity varies among studies (Table 1) and this could be due to interoperator variability in what constitutes LAF. It has been reported that there is a large spectrum of fluorescence intensity and a more definitive criteria of what constitutes LAF are required to reduce subjectivity and support the use of the device in wider clinical practice [24]. Furthermore, it has been suggested that a significant understanding of mucosal pathology is required to make correct clinical interpretations of VELscope findings [19], and this understanding may not be present in a general practice environment. Future research directions should evaluate the biological bases that contribute to false-positive and false-negative findings. If the specificity of the device could be improved, there would be an increased scope for the use of VELscope in routine general practice.

3. Identafi

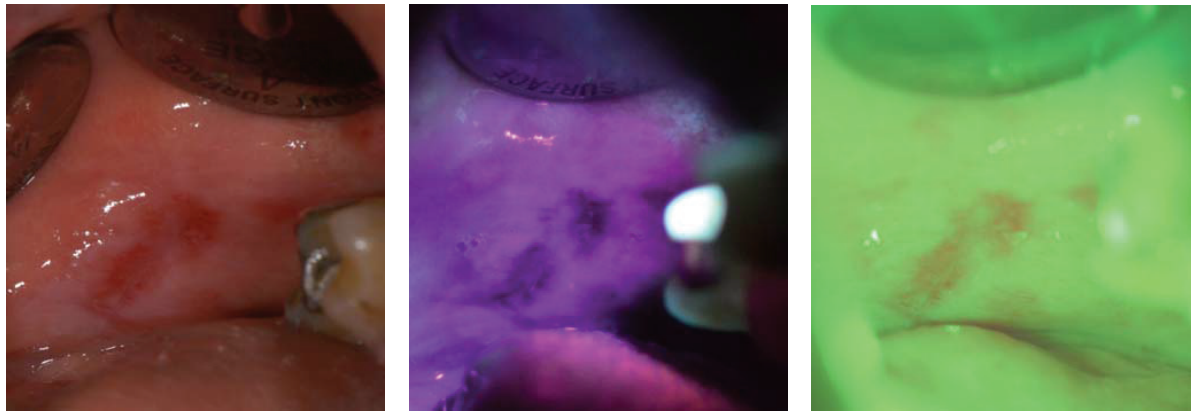
The Identafi (DentalEZ, PA, USA) is a multispectral screening device that incorporates three different lights which are designed to be used in a sequential manner to facilitate intraoral examination [44, 45]. In addition to a LED white light, Identafi also includes violet and green-amber lights to induce direct tissue fluorescence and tissue reflectance, respectively. Although previous research found that white light allows superior visualisation of oral mucosal lesions compared to routine incandescent light [46], differentiating between OPMDs and normal mucosa is still difficult with white light alone. By integrating tissue fluorescence and tissue reflectance into the one device, Identafi aims to be an easy to use device maximising the advantages of both white light examination and tissue fluorescence.

Violet light 405 nm in wavelength is used to assess, through the accompanying photosensitive filter glasses, the autofluorescence properties of oral tissues. As with VELscope, normal mucosa exhibits natural fluorescence, whereas abnormal tissues appear dark due to diminished autofluorescence or LAF (Figure 2). A study by Roblyer et al. [28] reported that light at 405 nm was the optimal excitation wavelength for discriminating between normal oral mucosa and dysplasia or OSCC, as it had 96 to 100% sensitivity and 91 to 96% specificity. However, a study by Sweeny et al. [47] involving 88 patients with a history of head and neck cancer reported 50% sensitivity and 81% specificity for Identafi's violet light and 50% sensitivity and 98% specificity for COE. The authors suggested radiation-induced changes such as fibrosis and pigmentation as possible causes for the low sensitivity. Another contributing factor was the lack of histopathology, as biopsies were not taken for every lesion, so it is possible that some areas with LAF had underlying dysplasia which was not apparent clinically. Results from this study must also be interpreted with caution as there was no indication whether or not OED, OSCC, or both were considered as positive findings. Nonetheless, preliminary cases by Lane et al. [21, 48] noted that areas of LAF were often larger than the clinically visible cancer when observed with violet light. They attributed this to the visualisation of deeper neovascularisation and stromal changes which accompany lesion progression, and therefore

Table 2: Published papers on the use of VELscope in general practice.

Purpose	Paper	Type of study	Sample population	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Notes
Used to detect OPMDs and/or oral cancer	Huff et al. [41]	Parallel cohort study	959 patients presenting to a private practice over a 12-month period received COE only. 905 presenting to the same private practice over a separate 12-month period received COE and VELscope examination.	—	—	—	—	—	For the COE only cohort, there was a 0.83% prevalence of mucosal abnormalities, with none being potentially malignant. For the combined COE and VELscope examination cohort, there was a 1.3% prevalence of mucosal abnormalities, with 83% of these being potentially malignant.
	Truelove et al. [42]	Prospective cohort study	620 patients seeking routine or emergency dental treatment at a dental school received both COE and VELscope examination.	—	—	—	—	—	Patients initially examined by dental students before the attending faculty. 5 dysplasias detected with VELscope were not found by COE.
	McNamara et al. [43]	Prospective cohort study	130 consecutive patients presenting to a screening clinic for routine dental care received both COE and VELscope examination.	67*	6*	6*	67*	—	No abnormalities detected with VELscope that were not found by COE. 93.8% (30/32) false-positive rate. 1 false-negative case. VELscope findings statistically different from scalpel biopsy gold standard ($P = 0.0001$).

*Calculated based on provided values.
OPMDs: oral potentially malignant disorders; COE: conventional oral examination.



(a) (b) (c)

Figure 2: Oral lichen planus on left buccal mucosa visualised with Identafi using its white light feature (a). The same lesion displaying loss of autofluorescence when visualised under violet light with Identafi (b), and microvasculature of the lesion is highlighted with the green-amber light (c).

proposed that this technology could assist in determining surgical margins for the excision of lesions.

The green-amber light at 545 nm wavelength utilises the concept of reflectance spectroscopy to delineate the vasculature in the connective tissue (Figure 2). Reflectance spectroscopy uses light within the absorption spectrum of haemoglobin—namely, between 400 and 600 nm—to visualise the underlying vasculature [49]. A significant reduction in the reflectance spectra of OSCCs and OPMDs occurs at 577 nm and 542 nm, and this is attributed to increased light absorption from increased microvasculature density and oxygenated haemoglobin concentrations in neoplastic tissue. Angiogenesis is an early step in carcinogenesis and a significant increase in microvessel count occurs in mild and moderate dysplasia [50–52]. Existing evidence also indicates that tumour-induced angiogenesis results in altered vascular morphology, and the degree of change can assist with determining the prognosis of oral lesions [50, 51]. This suggests that assessment of tissue angiogenesis in oral mucosal lesions enables the clinician to differentiate OPMDs from benign lesions. To date, however, there are very few published reports regarding the use of the 545 nm green-amber light. While the green-amber light was effective for highlighting the superficial vasculature and enhancing the keratinization of lesions in one study [21], another study [47] reported 0% sensitivity as no true-positive findings were detected. However, the authors acknowledged the low power of the study and that further research was required.

The publications thus far place little emphasis on the importance of the white light function of the device, and at this stage there is limited evidence to suggest any advantage of Identafi over COE. Results cannot be generalised to general practitioners as screening clinicians in these studies had specialist level training [21, 47]. Furthermore, there are currently no published clinical trials on the routine use of Identafi in the general population which were not funded by the manufacturer of the device. The ability for Identafi to differentiate between low- and high-risk lesions remains undetermined with the existing literature. There are currently several ongoing clinical trials [53], including several in our group, and until complete results from further research is published, use of the Identafi as a visualisation adjunct for OPMDs and OSCCs can only be justified based on our knowledge of comparable optical fluorescence imaging devices.

4. Narrow Band Imaging

Narrow band imaging (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic visualisation technology which enhances the mucosal surface texture and underlying vasculature by utilising the concept that the wavelength of light determines the depth of penetration [54, 55]. Two modes, white light and NBI, are included in the system to provide real time noninvasive optical image enhancement of mucosa. Switching between the two modes is simply achieved by pressing a button on the video endoscope, camera head, or system processor [56]. In NBI mode, filters placed in front of the white light allow only blue light between 400 and 430 nm (centred at 415 nm) and green light between 525 and 555 nm (centred at 540 nm) to be emitted simultaneously. Blood vessels in the superficial mucosa appear brown as the blue light penetrates shallowly and corresponds to the peak absorption spectrum of haemoglobin. Conversely, the green light penetrates deeper to highlight thicker blood vessels in the submucosa, and these vessels appear cyan [54, 55]. Reflected light is captured by a charge coupled device (CCD) located at the tip of the endoscope and is reconstructed by an image processor into a coloured composite image that is then displayed on a high-definition monitor screen [56]. In addition to the excellent resolution that can be maintained up to 2 mm away from the mucosa due to the physical zoom property, further enhancement of the mucosal texture and microvascular structures is possible with magnifying endoscopy [55, 57]. Up to 80 times optical magnification is available with the 2-band red-green-blue sequential NBI systems (e.g., Evis Lucera 260 Spectrum), whereas 1.2 times

digital zoom and 1.5 times digital zoom are available with the coloured CCD systems (e.g., Evis Exera II and Evis Exera III).

As previously stated, potentially malignant and malignant lesions have distinct microvascular morphology as angiogenesis is an early occurrence in carcinogenesis [58–60]. Neoplastic lesions appear as areas with scattered spots with a well-demarcated border, and can therefore be differentiated from inflammatory lesions which have an ill-demarcated border [61, 62]. These brown spots represent superficial vessels such as the intrapapillary capillary loops (IPCL). Visualisation of the vasculature gives clinicians a better idea of the true extent of lesions, and can therefore guide the position of biopsy and resection margins [61, 63, 64]. Furthermore, changes in the degree of dilation, meandering, tortuosity, and calibre of IPCLs indicate the severity of pathology present [58, 59, 61].

Oral lesions may be classified using Takano et al.'s [61] IPCL classification for oral mucosa based on the most advanced IPCL pattern present. Type I IPCL pattern is characterised by regular brown dots when loops are perpendicular to the mucosa or waved lines when parallel [61]. Although Type I IPCL pattern is typically associated with normal mucosa [61], a study by Yang et al. [65] involving 154 patients with newly diagnosed leukoplakia reported 17% frequency of dysplasia in lesions displaying this pattern. Therefore, clinicians should still retain a degree of suspicion and use clinical judgement when examining leukoplakia with Type I IPCL pattern. By contrast, Type II which has dilated and crossing IPCLs, and Type III which displays elongated and meandering IPCLs [61] are more frequently associated with dysplasia [65, 66]. While Type II is usually associated with nonneoplastic and inflammatory lesions, Yang et al. [65] reported 92% frequency of dysplasia for leukoplakia with this pattern, and Type III IPCL pattern had 100% frequency of dysplasia. These findings are supported by another study [66] which described similar classes of microvascular patterns. In this study [66], the IPCL Types II and III equivalents were associated with OPMDs and carcinoma. Type IV IPCL pattern, however, is indicative of neoplasia [65] and is characterised by large vessels IPCL pattern destruction and angiogenesis (Figure 3) [61]. Any lesion with Types III and IV should therefore be biopsied [67], particularly since the use of Types III and IV as the criteria for differentiating high-grade dysplasia, CIS, and invasive carcinoma from normal mucosa has been shown to have 85% sensitivity, 95% specificity, 74% positive predictive value (PPV), 97% negative predictive value (NPV), and 93% accuracy (Table 3) [65].

The efficacy of NBI primarily depends on light penetrating the epithelium to enhance the vasculature. A study by Lin et al. [70] found that areas with nonkeratinized thin stratified squamous epithelium had a significantly higher prevalence of brownish spots than areas with keratinized epithelium or epithelium thicker than 500 μm. However, Yang et al. [65] reported that the degree of keratinization did not affect visualisation of the underlying vasculature unless hyperkeratosis associated with leukoplakia was present. Visualisation of the microvasculature is possible through thin homogenous leukoplakia, but the vasculature will appear vague, blurry, or be completely obstructed where there is thick homogenous leukoplakia [67]. In the latter case, the IPCL pattern of the surrounding mucosa is often observed to guide the determination of the lesion's IPCL class; however, this is not completely reliable as one study [67] found dysplasia in 28% of thick homogenous leukoplakia surrounded by IPCL Type I. Instead, the degree of hyperkeratinization may be indicative of the degree of dysplasia, as IPCL Type I was only found beneath thin homogenous leukoplakia, whereas Types II and III were observed around thick homogenous leukoplakia.

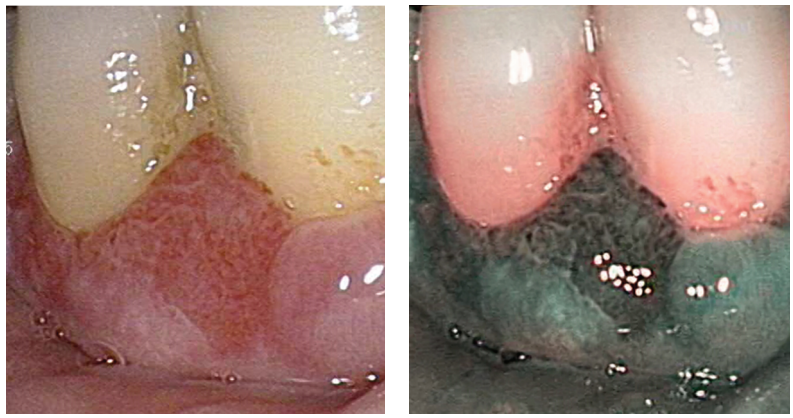
To date, there are only a few papers that have evaluated the use of NBI in just the oral cavity. The sensitivity, specificity, PPV, NPV, and accuracy for detecting oral neoplasia with NBI ranged from 95 to 96%, 97 to 100%, 91 to 100%, and 93 to 99% and 97%, respectively (Table 3) [68, 69]. In comparison, the ranges for white light were generally lower at 51 to 64%, 96 to 100%, 82 to 100%, 87 to 90%, and 68 to 89%, respectively. Chronic inflammation and chronic post-radiotherapy changes contributed to false positives, and this may be compounded by operator inexperience in recognising the different IPCL changes associated with inflammatory and neoplastic lesions. Regardless, NBI has great potential as a valuable adjunct to COE as it can detect malignancies that might otherwise be missed with white light.

There is currently no published clinical trial evaluating the efficacy of NBI for specifically detecting OPMDs in patients without oral cancer. Nonetheless, Nguyen et al. [72] conducted a prospective study involving 73 patients with head and neck cancer and found that the sensitivity for detecting moderate dysplasia or worse was at 96% with NBI, which was better than white light which had only 38%. It is possible that the efficacy for detecting dysplasia in OPMDs with NBI will be similar to that noted for OSCCs.

Although the use of NBI as an adjunct to COE for detecting OMPDs and OSCCs shows promise, the literature is still very limited. Results from published papers cannot be generalised to the general population as all studies have been conducted in specialist settings. In addition, NBI is only intended for use in secondary and tertiary settings due to the cost of the technology and training required. More prospective clinical trials are required to evaluate the efficacy of NBI for aiding the detection and surveillance of OPMDs and OSCCs.

5. Limitations of Optical Aids

The literature indicates that optical aids are effective in highlighting oral mucosal abnormalities but cannot effectively differentiate between those which are considered "low risk" or "high risk." With VELscope and Identafi, this can be attributed to the biological basis which contributes to LAF of the oral mucosa. As previously discussed, LAF is a product of alterations in the metabolic activity in the epithelium and changes to the collagen architecture in the stroma [27]. Haemoglobin also has a significant influence on autofluorescence spectra of malignancies [73]. During oncogenesis, there is increased cellular proliferation resulting in increased metabolic activity combined with architectural changes in the stroma and angiogenesis, all of which contribute to LAF [27, 29, 50–52]. Benign inflammatory lesions such as oral lichen planus display increased vascularity and inflammation which introduces haemoglobin into the tissues and thus contributes



(a) (b)

Figure 3: SCC of the gingiva viewed with endoscopic white light (a). Same lesion viewed in NBI mode demonstrating Type IV IPCL pattern (b).

to LAF. Vascular lesions also display LAF due to the increase in local haemoglobin content in the vasculature. In addition to this, not all dysplasias, particularly those at an early stage, display LAF [19]. Clinically, it cannot be determined if LAF is due to neoplasia or to benign inflammatory origin; therefore, autofluorescence alone cannot discriminate between "high-risk" and "low-risk" lesions, and careful clinical correlation is required. A similar dilemma exists for NBI as use of the technology is primarily based on clinically analysing the morphology of the underlying microvasculature. While applying a defined classification system is beneficial for interpreting IPCL patterns, it is still a subjective method and is not infallible as each class of IPCL pattern does not always correspond to a particular histopathological diagnosis [65]. Further complicating the matter is keratosis, which prevents clear visualisation of the underlying microvasculature [67]. Abnormal microvasculature patterns associated with chronic inflammation or vascular lesions can also act as confounders when using NBI [69]. Therefore, while these visualisation adjuncts can demonstrate the presence of an abnormality, they cannot effectively differentiate between "high-risk" and "low-risk" lesions in their current state.

6. Other Approaches to Optical Imaging

6.1. Autofluorescence with Endoscopic Techniques. In order to improve the accuracy of optical imaging techniques, different technologies have been combined into multimodal imaging devices. One example is the combination of autofluorescence imaging with a high-resolution microendoscope system (HRME). While autofluorescence can be used for examination of a wide field, HRME is designed to assess specific sites. Pierce et al. [74] found increased sensitivity and specificity for the detection of OED and OSCC by combining autofluorescence imaging and a high-resolution microendoscope system compared to either technology alone.

Another example is the use of NBI with fluorescence imaging. A prospective study by Nguyen et al. [72] assessed the use of autofluorescence and NBI on patients with a history of SCC in the head and neck. Sensitivity for detecting dysplasia was higher with autofluorescence (96%) and NBI (96%) than with white light (37%), and high specificity was noted for the combined use of autofluorescence and NBI. The addition of autofluorescence and NBI to white light endoscopic examination also influenced the management of 6% of patients. Similar results were reported in a study that assessed for dysplasia in patients with Barrett's oesophagus using the combination of fluorescence imaging, reflectance, and light scattering microscopy. In this study, the sensitivity and specificity were 93% and 100%, respectively, when at least two of the three lights indicated neoplasia [75]. The addition of NBI following autofluorescence imaging in the surveillance of Barrett's oesophagus also reduced the false-positive rate of autofluorescence from 40% to 10% without affecting sensitivity [76].

An alternative combination commercialised for use in the oesophagus is the endoscopic trimodal imaging (ETMI) system which incorporates white light endoscopy, autofluorescence imaging, and NBI. As with the previous study, the use of NBI following autofluorescence imaging removed some false-positive findings; however, it also misclassified areas of neoplasia [77]. While ETMI is more effective than targeted biopsies in the surveillance of Barrett's oesophagus, the overall histological yield is greater through the use of standard video endoscopy with both random and targeted biopsies [77].

6.2. Fluorescence Lifetime Imaging. Another potentially useful technology is fluorescence lifetime imaging, which assesses the decay of fluorescence [78, 79]. Following excitation, autofluorescence is emitted for up to ten nanoseconds and during this time, the decay in fluorescence can be measured [80]. It is suggested that fluorescence lifetime imaging is unaffected by excitation intensity, fluorophore concentrations, or attenuation due to tissue absorption or scattering [79]. Although laser guided fluorescence provides promise as a diagnostic tool that can act as an "optical biopsy", it is impractical for screening purposes where wide-field autofluorescence visualisation is desirable. Consequently, Galletly et al. [79] devised a method using fluorescence lifetime

Table 3: Published papers on the use of NBI in the oral cavity.

Paper	Purpose	Type of study	Sample population	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Notes
Katada et al. [63]	Used to detect superficial SCC	Case report	2 patients with oesophageal cancer.	—	—	—	—	—	Coincidental finding of OSCC at floor of mouth.
Piazza et al. [68]	Used to detect dysplasia and SCC	Prospective cohort study	96 patients with biopsy-confirmed or previous-treated OSCC or oropharyngeal SCC.	96	100	100	93	97	Combined the use of NBI with high-definition television. 27% (26 of 96) patients had a diagnostic benefit with the use of NBI and high-definition television.
Takano et al. [61]	Investigated the types of IPCL patterns	Prospective cohort study	41 patients with normal mucosa, or nonneoplastic or neoplastic lesions.	—	—	—	—	—	Devised the IPCL classification of oral mucosa.
Chu et al. [69]	Used to detect dysplasia and OSCC	Prospective cohort study	101 patients with treated OSCC.	95%	97%	91%	99%	97%	Had difficulty diagnosing hyperkeratotic lesions, tumours at the tongue base, and recurring tumours. Chronic inflammation, postoperative radiation, colouration, or mucosal staining interfered with diagnosis.
Lin et al. [70]	Investigated the visibility of brownish spots in different types of epithelium	Prospective cohort study	125 patients with CIS or SCC in the head and neck.	—	—	—	—	—	Areas with nonkeratinized thin stratified squamous epithelium had a significantly higher prevalence of brownish spots than areas with keratinized epithelium or epithelium thicker than 500 μm.
Tan et al. [64]	Used NBI to influence management of oral erythroplakia	Case report	1 patient with erythroplakia.	—	—	—	—	—	NBI was used to determine resection margins, which were beyond the clinically visible margins.

Table 3: Continued.

Paper	Purpose	Type of study	Sample population	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Notes
Yang et al. [65]	Correlated NBI clinical findings with histopathology	Retrospective cohort study	154 patients with oral leukoplakia.	—	—	—	—	—	The IPCL classification had a significant statistical association with the severity of pathology.
Yang et al. [67]	Evaluated the use of NBI for assessing and managing oral leukoplakia	Retrospective cohort study	160 patients with clinical homogenous oral leukoplakia.	—	—	—	—	—	All cases of thin leukoplakia had IPCL Type I and were confirmed as squamous hyperplasia. Thick leukoplakia had IPCL Type I, II, or III, and a significant correlation between pathology and NBI images was present ($P < 0.0001$).
Yang et al. [71]	Used IPCL patterns made visible by NBI to diagnose high-grade dysplasia, CIS, and OSCC	Retrospective case-control study	414 patients with oral leukoplak.ia	15%	60%	7%	79%	53%	Criteria used was "brownish spots and demarcation line with irregular microvascular pattern".
				77%	55%	24%	93%	58%	Criteria used was "well-demarcated brownish area with thick dark spots and/or winding vessels".
				85%	95%	75%	97%	93%	Criteria used was "the intraepithelial papillary capillary loop (IPCL) Type III . . . and Type IV".
Yang et al. [66]	Investigated the IPCL morphology of OSCC and correlated the pattern with infiltration depth and disease severity	Retrospective cohort study	80 patients with-biopsy confirmed OSCC	—	—	—	—	—	The IPCL pattern moved from tortuous and dilated to twisted and elongated to angiogenesis and destruction of IPCL as the severity of OSCC increased. Depth of infiltration increased with the degree of severity.
Nguyen et al. [72]	Compared white light, autofluorescence, and NBI to detect moderate dysplasia or worse	Prospective cohort study	73 patients with known or treated head and neck SCC	96	79	85	94	—	Autofluorescence and NBI were significantly more sensitive than white light. NBI and white light were more specific than autofluorescence.

NBI: narrow band imaging; OSCC: oral squamous cell carcinoma; SCC: squamous cell carcinoma; IPCL: intrapapillary capillary loop; and CIS: carcinoma in situ.

imaging which allows for a large field of view. Therefore, this could be used for both detecting small or poorly visible lesions and for delineating surgical margins for excision [79].

In the oral cavity, OPMDs are associated with increased fluorescence decay times [81]. This model utilised a 410 nm wavelength and measured emission at the 633 nm wavelength and much like previous studies calculated a threshold value to classify lesions. Chen et al. [81] were able to accurately distinguish cases of verrucous hyperplasia and OED from normal mucosa using this approach.

6.3. Optical Coherence Tomography. Optical coherence tomography (OCT) is an optical imaging modality which utilises low-power infrared light between 750 and 1300 nm and a Michelson interferometer to produce high-resolution, cross-sectional, and subsurface tomographic images of tissue microstructure [82, 83]. Interaction of the light with the tissue surface causes scattering, and images are generated by measuring the echo time delay and the intensity of backscattered light [84, 85]. The depth of penetration for OCT imaging is approximately 1 to 3 mm depending on the tissue structure, depth of focus of the probe used, and pressure applied to the tissue surface [86].

OCT has been profoundly used in ophthalmic practice to provide *in vivo* "optical biopsy" of the retina [87], in addition to dermatological applications which include evaluation of skin tumours [88], and inflammatory disease [89].

OCT can be used to identify architectural changes in the keratin cell layer, epithelial layer, basement membrane, lamina propria, and rete pegs of oral mucosa [90]. Although OCT is capable of assessing lesions for neoplastic changes by determining the thickness of the epithelial layer, integrity of the basement membrane, and changes in the lamina propria [91], it is still incapable of providing enough cellular information to grade OPMDs [90]. Early research revealed that while OCT imaging displayed 93% sensitivity and 97% specificity for diagnosing OSCCs when compared to histology in one study [92], another study reported that its ability to differentiate between different oral mucosal abnormalities was poor [91]. Recent research reflects the latter finding, with sensitivity and specificity of 85% and 78%, respectively, for the identification of OPMDs using *ex vivo* biopsies [93]. Tsai et al. have analysed OCT profiles for the delineation of OSCC margins and suggested that this could be used to develop an algorithm for the detection and delineation of OSCCs [94, 95]. Further research is required on the potential application of OCT to improve and define excisional margins during surgical management of OPMDs and OSCCs.

6.4. Angle-Resolved Low-Coherence Interferometry (a/LCI). Angle-resolved low-coherence interferometry (a/LCI) is another noninvasive optical spectroscopic technique which, like laser induced autofluorescence and OCT, can be used to perform an "optical biopsy" of intact, living tissue [96, 97]. a/LCI utilises measurements of angular light scattering of cellular nuclei to calculate the average nuclear diameter, and like OCT, this method can provide information as a function of depth of epithelium [96, 97]. The detailed science behind a/LCI has been covered elsewhere [97].

Differences in the average nuclear diameter can be used to differentiate dysplasia from normal tissue, as increased nuclear diameter is associated with neoplastic progression [98–101]. Wax et al. [100] created an algorithm for the use of a/LCI by calculating threshold values for dysplasia with a rat oesophageal carcinoma model, and on prospective analysis, this technique proved to be highly accurate with sensitivity and specificity of 91% and 97%, respectively [101]. The advantage of this technology over other technologies such as autofluorescence or NBI is that it allows for targeted sampling through depth sensitivity analysis. Using a/LCI on human biopsy tissue, Brown et al. [102] confirmed that analysing deeper tissue segments near the basal layer, when compared to more superficial layers, provided the highest degree of accuracy for observing dysplasia and hence had the greatest diagnostic potential.

Zhu et al. [103] created a prototype a/LCI system which was programmed to assess, through an endoscope, the mean nuclear diameter of oesophageal tissue cells located at 200 to 300 μm in depth, near the basal layer. Forty-six patients undergoing routine endoscopic screening for Barrett's oesophagus were assessed using this prototype system to obtain an "optical biopsy" which was then compared with histology [98]. The determined threshold value for this model had 100% sensitivity, and specificity of 84% when all samples were included and 85% when only samples with Barrett's oesophagus were considered [98]. Terry et al. [104] found that a/LCI was able to diagnose dysplasia on *ex vivo* samples of colonic tissue with 85% accuracy and suggested that this technique may be ready for *in vivo* studies for the diagnosis of intestinal dysplasia.

While research is at an early stage, a/LCI appears to be a highly accurate method of diagnosing dysplasia during routine screening of patients with Barrett's oesophagus. Larger-scale trials are required to determine the most appropriate threshold value and whether this technology can replace the need for physical biopsies. There are currently no published papers regarding the use of a/LCI in the oral cavity; however, the use of this technology may reduce the number of physical biopsies through its potential ability to provide on-the-spot diagnosis of lesions.

6.5. Use of Algorithms. As previously discussed, a severe limitation of existing optical imaging techniques is the arbitrary nature of interpretation leading to a high level of interoperator variability. To reduce this, a newer approach targeted towards creating algorithms based on quantitative data allows for computer analysis of the fluorescence properties and thus removes operator bias. Such models have been established with a high degree of accuracy for the diagnosis of oesophageal cancer using laser-induced autofluorescence at 410 nm excitation wavelength [105–107]. Vo-Dinh et al. found that at this excitation wavelength, oesophageal malignancies were associated with reduced fluorescence intensity and changes in fluorescence spectra [105, 106]. While malignancies were associated with less fluorescence intensity, the intensity was found to be an inconsistent parameter and a more independent measurement was required. Instead, the group assessed the fluorescence spectra of normal and malignant

tissue between 430 and 720 nm and normalized for intensity to remove variations between lesions. The most significant differences in differential normalized fluorescence (DNF) in malignancies were found at 480 nm and 660 nm wavelengths [105, 106]. Initial evaluation found this model could differentiate malignant tissue from normal tissue with greater than 98% accuracy [105, 106]. Such an approach removes the operator from the decision-making process and allows for simple computer-based classification of lesions into benign and malignant. Due to the high accuracy of this technique, it may be used as an "optical biopsy", thereby significantly reducing the need for a physical biopsy and the associated morbidity, time, and financial costs [106]. When this technique using DNF data was extended to assess for high-grade dysplasia in Barrett's oesophagus, it was associated with a high specificity and a high sensitivity [108]. In this study however, low-grade dysplasia was considered benign as no surgical intervention was required. Of concern, only 28% of low-grade with focal high-grade dysplasias were considered positive [108]. This may be due to the endoscopic laser only being able to assess small areas of tissue at a time, while the biopsy sample may include adjacent tissue with foci of high-grade dysplasia. Unlike direct tissue fluorescence visualization, the presence of inflammation does not create false-positive findings using DNF indices [109]. A similar approach could be utilised in the oral cavity to increase the sensitivity of autofluorescence visualisation. If molecular markers associated with LAF could be found, it is possible to target these by identifying their associated fluorescence spectra.

To reduce the effect of operator bias, Roblyer et al. [28] introduced an objective method of distinguishing oral neoplasia from benign tissue using autofluorescence with a 405 nm light source. An algorithm was created on a test group of patients presenting with oral mucosal lesions as well as volunteers with healthy mucosa. The authors found a reduction in green fluorescence and an increase in red fluorescence to be highly associated with neoplastic tissue. They determined that the normalized red-to-green ratio was able to predict the risk of neoplasia and calculated a threshold value beyond which the area would be considered suspicious for neoplasia. Using the images obtained under 405 nm excitation, a probability map was generated by calculating the risk of neoplasia for each area of tissue, and areas with a risk of dysplasia being greater than 50% were highlighted. In the training group, this algorithm was associated with a sensitivity and specificity of 96%, whereas in the validation group, these values were 100% and 91%, respectively. As with the approach taken in oesophageal cancers, this method uses direct tissue autofluorescence but removes the factor of operator bias. Future research should examine the construction of a simple device which could create similar probability maps chairside, as this would improve the usefulness of direct tissue fluorescence in helping determine biopsy location and surgical margin delineation.

7. Conclusions

Detection of OPMDs before they advance to OSCC is predicted to improve survival rates for oral cancer. Evidence indicates that COE is a poor discriminator of oral mucosal lesions, and this has led to the development of several adjunctive visualisation aids. VELscope is associated with high sensitivity and can assist in the detection of additional lesions; however, additional research is required to reduce the incidence of false positives. Further research is also required to assess the efficacy of Identafi, a multispectral device with limited available scientific and clinical literature. NBI shows great promise as a useful adjunct to COE, as several studies have reported that it performed better than white light at detecting malignancies. However, there is currently no published clinical trial evaluating the efficacy of NBI for detecting OPMDs in patients who do not have confirmed oral cancer. Therefore, before these visualisation adjuncts can gain widespread use, larger well-designed prospective studies for each technology are required. Further studies are also required into the molecular basis for fluorescence imaging to help determine the factors contributing to false-positive and false-negative findings. Research into the biological mechanisms of angiogenesis associated with oral cancer will hopefully provide a clearer understanding of the microvascular changes that occur in OPMDs and OSCCs. This in turn may lead to more effective and predictable methods for accurately interpreting NBI data. Future approaches to optical imaging could involve real time quantitative evaluation to determine a diagnosis for oral mucosal lesions rather than simply highlighting the presence of abnormalities, thus, making the possibility of "optical biopsy" a clinical reality.

Conflict of Interests

Camile S. Farah is the Guest Lead Editor of the Special Issue on Oral Cancer and Oral Potentially Malignant Disorders for the International Journal of Dentistry. This paper has undergone independent external peer review and has been handled by one of the other guest editors. Camile S. Farah has evaluated VELscope, Identafi, and NBI devices donated by their respective manufacturers for undertaking clinical research.

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Clinical Study

Clinical Outcome Following Oral Potentially Malignant Disorder Treatment: A 100 Patient Cohort Study

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Oral potentially malignant disorders (PMDs) are at risk of transforming to invasive squamous cell carcinoma (SCC), but controversy exists over their management and the precise role of interventional treatment. In this study, a cohort of 100 patients presenting with new, single oral dysplastic PMD lesions were followed for up to 10 years following laser excision. PMDs presented primarily as homogeneous leukoplakias on floor of mouth and ventrolateral tongue sites and showed mainly high-grade dysplasia following analysis of excision specimens. Sixty-two patients were disease-free at the time of the most recent followup, whilst 17 experienced same site PMD recurrence, 14 developed further PMDs at new sites, 5 underwent same site malignant transformation, and 2 developed SCC at new oral sites. Whilst laser excision is an effective therapeutic tool in PMD management, prolonged patient followup and active mucosal surveillance together with clear definitions of clinical outcomes are all essential prerequisites for successful interventional management. Multicentre, prospective, and randomised trials of PMD treatment intervention are urgently required to determine optimal management strategies.

1. Introduction

Oral potentially malignant disorder (PMD) is the preferred WHO term to describe a number of mucosal lesions which demonstrate an increased risk of squamous cell carcinoma (SCC) development compared with apparently normal oral mucosa. The list of mucosal pathology considered potentially malignant includes discrete lesions such as leukoplakia and erythroplakia, as well as more widespread conditions such as proliferative verrucous leukoplakia, immunodeficiency, oral submucous fibrosis, and perhaps more controversially oral lichenoid lesions [1].

Whilst a vast literature exists describing the aetiology, clinical appearance, and the identifiable histopathological features of dysplasia seen in PMD, there remain no universally agreed clinical management protocols. We have described previously, however, both the diagnostic accuracy of obtaining definitive histopathology specimens and the treatment efficacy of the entire lesion removal by interventional laser surgery, and it is now generally accepted that PMD excision is probably the optimal management option [1–3].

It remains impossible, unfortunately, to predict either the behaviour of individual PMD lesions or the progress of disease in a particular patient, and some authors raise concerns that formal PMD excision is not proven to prevent SCC development, although it remains a not unreasonable hypothesis [2, 3].

Of perhaps more significance is the lack of clarity regarding overall clinical outcome following PMD treatment and a need to both rationalise terminology and define a more structured patient follow-up regime. The aim of this paper, therefore, is to report on the detailed clinical outcome and followup of a cohort of 100 PMD patients who all underwent a standardised interventional laser surgery treatment to excise dysplastic single lesion disease and whose postoperative progress was documented for up to 10 years following the first presentation.

2. Materials and Methods

2.1. Patients. Following ethical committee approval and informed patient consent, 100 consecutive PMD patients

attending the Maxillofacial Oncology/Dysplasia clinics at Newcastle upon Tyne in Northern England over a 3-year period and who underwent CO_2 laser excision of dysplastic lesions were recruited to the study. All were new patients, with no prior history of oral cancer or precancer and no previous surgical or radiotherapy treatment, and all presented with distinct, single-site PMD lesions proven on incisional biopsy to exhibit dysplasia.

Laser surgery was carried out by the same operator (P. J. Thomason) working to a standardised protocol, which has been previously documented, and which comprised formal excision of mucosal lesions and widespread ablation of mucosal margins [2, 3]. The influence of risk factor behaviour such as smoking and alcohol use was identified and appropriate cessation advice was given prior to treatment. All patients were reviewed on a regular basis postlaser intervention, at varying intervals between 1 and 12 months based upon the severity of individual clinical and pathological features, to monitor the clinical course of disease and patients' outcome. The identification of new mucosal disease, biopsy for histopathological diagnosis, and further interventional treatment was carried out in accordance with defined management protocols [2, 3].

All excision biopsy specimens underwent standardised histopathology examination by two experienced oral pathologists (C. M. Robinson and P. Sloan) working to agreed diagnostic criteria. Lesions were graded using both the 2005 World Health Organisation (WHO) classification [4] and a binary grading system (high grade versus low grade) that benefits from increased levels of interobserver agreement and improved predictive value [5]. The two pathologists independently assessed the biopsy material, and discordant grading was resolved by review and consensus. The size of dysplastic lesions was assessed by multiplying the length by width of laser excised specimens as recorded in histopathology reports.

2.2. Clinical Outcome. Clinical outcome for each patient was defined at the time of their most recent followup appointment using one of the following terms: *Clinical Resolution*, a patient clinically free of PMD disease following treatment, *Persistent Disease*, whereby the PMD lesion persisted at the same site despite interventional treatment, *Recurrent Disease*, when a PMD lesion recurred at the same site following previously successful excision, *Further Disease*, distinguishing PMD lesion development at new oral sites following previously successful excision, *Malignant Transformation*, whereby invasive SCC arose at the same site of a clinically recognised oral precursor lesion, and *Oral Cancer Development*, in which invasive SCC development occurred but at new oral sites distant from previously recognised or treated precursor lesions.

2.3. Statistical Analysis. Statistical analyses were performed using SPSS, version 19.0 (Statistical Package for the Social Sciences, Chicago, IL, USA). Categorical variables for clinical outcome data were summarized and presented descriptively using frequencies and percentages with the chi-square test or Fisher's exact test used to evaluate relationship between variables. Continuous variables were expressed as mean ± standard deviation and were compared using independent Student's t-test for pairwise comparison; Kruskal-Wallis test was used for group comparison. Spearman's correlation was used to find and evaluate correlation between variables. The Kaplan-Meier survival analysis method with log-rank test was used to assess the differences between outcome groups and to calculate cumulative disease-free survival rates. For all tests, P values ≤ 0.05 were considered statistically significant.

Table 1: Anatomical site of PMD lesions.

Anatomical site	Number of lesions
Floor of mouth	46
Lateral tongue	19
Ventral tongue	14
Soft palate	9
Buccal mucosa	5
Fauces	4
Alveolus	2
Retromolar	1

3. Results

Sixty-eight male patients (age range 30–81 years; mean 58 years) and 32 female patients (age range 33–94 years; mean 59 years) comprised the study cohort. Eighty-six patients were either current or ex-smokers, whilst 83 regularly consumed alcohol. One hundred lesions were formally excised by laser, the majority (76) appearing clinically as leukoplakias (67 homogeneous and 9 nonhomogeneous), and the remaining were classified as erythroleukoplakias (16) and erythroplakias (8). Most lesions (79) presented on the floor of mouth and ventrolateral tongue, as summarised in Table 1.

Following consensus WHO grading of the excision specimens, 42 of the lesions were classified as mild dysplasia, whilst the remainder showed moderate dysplasia (26), severe dysplasia (21), or carcinoma in situ (11) (Kappa value = 0.644, $P < 0.001$). Consensus classification using the binary grading system confirmed 56 of the moderate, severe, and carcinoma-in-situ groups as "high grade" and 44 (42 mild and 2 moderate) as "low-grade" lesions (Kappa value = 0.756, $P < 0.001$).

Following laser excision of their PMD lesions, patients were reviewed for between 2 and 10 years with a mean followup of around 5 years. Nearly two-thirds of cases (62 patients) were completely disease-free following laser surgery, whilst 17 had developed recurrent (same site) disease, 14 further (new site) disease, 5 same site malignant transformation, and 2 developed SCC at new oral sites distinct from their original presenting PMD. No cases in this patient cohort exhibited persistent disease following laser excision.

A number of clinicopathological features were examined in detail for significance in relation to the documented clinical outcome for the 100 PMD patients.

3.1. Age. Chi-square testing revealed no significant relationship between patient age and observed clinical outcome ($P = 0.361$), although middle-age patients (41 to 62 years of age) were predominant in all outcome groups. No SCC development at new sites was seen in patients younger than 40 years.

3.2. Sex. Although more male patients presented with PMD lesions, there were no statistically significant associations seen between sex and treatment outcomes in this study ($P = 0.811$; chi-square test).

3.3. Clinical Appearance. The vast majority of lesions in this study were leukoplakias, and there were no significant clinical outcome differences discernible between these and lesions with erythroplakic or erythroleukoplakic appearance ($P = 0.234$, Fisher's exact test). Whilst clinical appearance was not significantly related to histopathological diagnosis, non-homogeneous leukoplakia did show higher rates for both recurrent and further dysplastic lesions compared to homogeneous lesions ($P = 0.016$, chi-square test).

3.4. PMD Site. Most lesions presented on the floor of the mouth and/or ventro-lateral tongue, with a significant relation seen between clinical outcome and anatomical site of origin ($P = 0.020$, chi-square test), whereby the majority of recurrent and further dysplastic disease cases were seen on the floor of the mouth and ventral tongue. The single dysplastic retromolar lesion in this study underwent malignant transformation, whilst new site SCC development only occurred in patients presenting initially with ventro-lateral tongue lesions.

3.5. PMD Lesion Size. A significant relation was found between clinical outcome and PMD lesion size, categorised as $<200\,mm^2$, between 200 and $600\,mm^2$ and $>600\,mm^2$ ($P = 0.010$, chi-square test). Clinical resolution was most commonly seen in minor and intermediate sized lesions. A higher mean size of presenting lesion was seen in patients developing recurrent disease ($393.63\,mm^2$) compared to recurrence-free ($281.70\,mm^2$), albeit nonsignificant ($P = 0.356$, independent t-test). Further (new site) dysplastic disease was significantly more common following intermediate sized precursor lesion excision ($P = 0.049$, chi-square test).

Although (same site) malignant transformation was more common following intermediate sized lesion excision, this was not statistically significant ($P = 0.593$, Chi-Square test). However, risk estimate showed that if initial dysplasia size exceeded or equalled $425\,mm^2$ (equivalent to the third quartile), the odds ratio for transformation was 2 times higher than that of smaller sized lesions (95% CI, 0.365–11.582).

SCC development distant from primary lesion sites was only seen in intermediate or major sized lesions, and although nonsignificant ($P = 0.104$, Fisher's exact test), there was a definite trend for lesions $>200\,mm^2$ to exhibit further disease, malignant transformation, and SCC development following treatment.

3.6. Smoking Behaviour. The vast majority of patients in this study were either current or ex-smokers, and a significant relation was found between smoking status and clinical outcome ($P = 0.014$, chi-square test), whilst the incidence of both recurrent and further disease was the highest in patients exposed to tobacco, there was a trend for nonsmokers to risk malignant transformation and particularly SCC development, whereby new site carcinomas were seen exclusively in nonsmokers.

Nonsmoking patients also presented with significantly larger lesions (mean size $473.20\,mm^2$), compared with both ex-smokers ($354.25\,mm^2$) and current smokers ($241.49\,mm^2$), $P = 0.026$, Kruskal-Wallis test. Also, Spearman correlation revealed a significant positive correlation between the degree of histopathological grading and lesion size ($r = 0.272$; $n = 96$; $P < 0.01$), whereby increased PMD size was associated with increased dysplasia severity.

Chi-Square testing, however, showed no significant relation between clinical outcome and the number of cigarettes smoked per day ($P = 0.139$).

3.7. Alcohol Use. There were no statistically significant relationships seen between alcohol intake and outcome ($P = 0.267$, chi-square test), although patients consuming regular alcohol posttreatment risked both recurrent and further disease development, whilst all 3 patients who ceased alcohol consumption remained disease-free.

3.8. Histopathological Grading. The WHO system showed a significant relationship with defined outcome categories ($P = 0.003$, Chi-Square test); patients exhibiting malignant transformation or new site SCC development displayed were those seen with either severe dysplasia or carcinoma-in-situ in presenting PMDs.

Recategorising clinical outcome as either clinical resolution (disease free) or further disease (encompassing recurrent/further PMDs, malignant transformation, or SCC development) emphasised that lesions with severe dysplasia and carcinoma-in-situ were statistically more likely to develop further disease ($P = 0.010$, chi-square test). The degree of dysplasia also had a significant effect on unfavourable outcome, with severe dysplasia/carcinoma-in-situ having a shorter mean time to develop further disease (40 months) compared with moderate (78.8 months) or mild dysplasia (87.83 months). Also, 2- and 5-year disease-free survival rates were much lower for severe dysplasia/carcinoma-in-situ than for either moderate or mild dysplasia (63%, 76%, and 85% and 14%, 59%, and 62%, resp.), $P = 0.006$, Log-Rank test. These data are presented in Figure 1.

In terms of binary grading, there were demonstrably more high-grade lesions in the outcome groups of recurrent, further disease, malignant transformation, and SCC development, but this was statistically significant only for recurrent disease ($P = 0.025$, Fisher's exact test). Increased statistical significance was seen, however, by recategorising outcome as either clinical resolution or further disease, confirming an increased risk of further disease with high-grade lesions ($P = 0.021$, chi-square test). Patients with high grade dysplasia

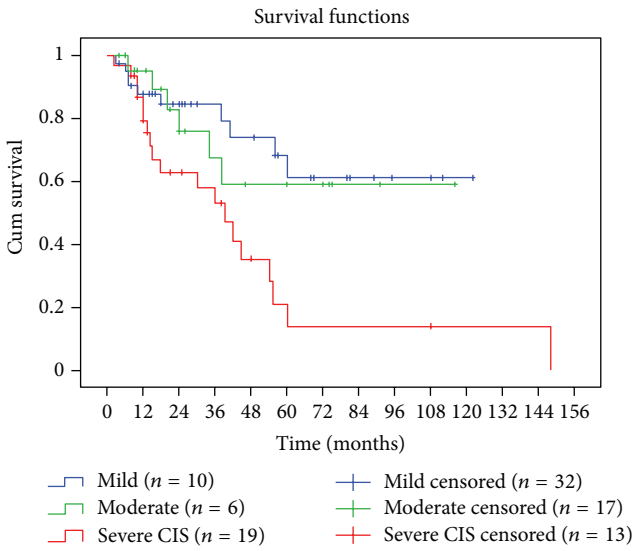

Figure 1: Kaplan-Meier analysis plotting disease-free survival according to the WHO grading of dysplasia ($P = 0.006$, Log-rank test).

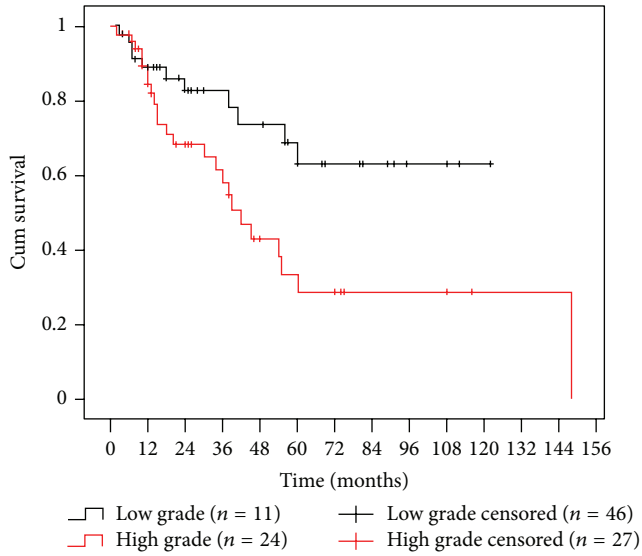

Figure 2: Kaplan-Meier analysis plotting disease-free survival according to high- and low-grade dysplasia ($P = 0.013$, Log-Rank test).

also had a significantly shorter mean time (64 months) to develop an unfavourable outcome compared to low-grade lesions (88.7 months), and lower 2- and 5-year disease-free survival rates were seen for high-grade compared to low-grade dysplasia (68% versus 83% and 29% versus 63%, resp.), $P = 0.013$, Log-Rank test. These data are summarised in Figure 2.

3.9. Laser Excision Margin Analysis. Fourty eight PMD excision specimens had clear margins on histopathological examination with no discernible dysplastic features, whilst in 23 cases foci of mild dysplasia were identified; less commonly, moderate (14) and severe dysplasia (12) or rarely foci of carcinoma-in-situ (3) were reported. Additional intervention for dysplasia positive margin cases was not usually required due to active ablation of all margins at the time of laser excision, although all cases underwent careful clinical surveillance.

Whilst the presence of dysplasia in excision margins did not significantly influence overall postlaser surgery clinical outcome ($P = 0.053$, Fisher's exact test), the majority of patients free from either recurrent (same site) disease or further (new site) disease had clear resection margins, whilst those developing further disease primarily exhibited severe dysplasia in excision margins ($P = 0.004$ and $P = 0.050$, resp., chi-square test).

3.10. Length of Followup. Clinical outcome in relation to length of followup was determined by plotting PMD-free survival via Kaplan-Meier survival analysis, and this showed a clear relationship with time. Figure 3 confirms that whilst 88 patients exhibited clinical resolution and were disease-free 1 year after surgery, there was a progressive rise in recurrent and further disease through successive years so that disease-free rates fell to 75 at 2 years, 68 at 3 years, and 47 at 5 years, with only 42 patients PMD-free 10 years after surgery.

Figure 4 shows that recurrent (same site) PMDs most commonly presented during the first 2 years following laser surgery (11 out of 17 cases), $P = 0.0001$, Log-Rank test. In contradistinction to recurrence, Figure 5 illustrates that further (new site) PMD disease could arise at any time during the first five years of followup, but with particularly significant risk at 1 and 3 years after surgery, $P = 0.0001$, Log-Rank test.

Five malignant transformation cases (same site) occurred during the first 15 months of followup ($P = 0.0001$, Log-Rank test), Figure 6. SCC development (new site cancer) only occurred in 2 cases, both nearly 5 years following severe dysplasia excision from the ventro-lateral tongue.

3.11. Risk Profiling. The clinico-pathological profile of PMD cases observed in each clinical outcome category is summarised in Table 2. Further statistical analysis was performed using univariate and multivariate logistic regression analysis to predict the role of patient age, sex, lesion size, type, histopathology, anatomical site, and resection margin status upon unfavourable clinical outcome (disease active state including recurrent or further dysplasia, malignant transformation, and OSCC development), Table 3.

Whilst patients' age and sex showed no significant effects, non-homogenous leukoplakia was a significant predictor of active disease ($P = 0.023$), increasing risk by nearly 3 times compared to homogenous lesions. Tongue lesions showed a 3.4 increased risk compared with floor of mouth ($P = 0.013$). The presence of severe dysplasia was a highly significant predictor of active disease status ($P = 0.007$); severe dysplasia and carcinoma-in-situ showed a 4.6 and a 4.8 times increased risk, respectively, for active disease compared

Table 2: Risk profile and clinical outcome.

	Clinical resolution	Recurrent disease	Further disease	Malignant transformation	OSCC development
Number of cases	62	17	14	5	2
Sex					
Male/female	39/23	13/4	10/4	3/2	1/1
Age (Yrs)					
Mean (range)	57 (33–71)	58 (40–77)	59 (39–76)	63 (58–76)	(47–48)
Lesion size (mean mm^2)	Minor (251)	Major (394)	Major (343)	Intermediate/major (361)	Major (478)
Pathology grading (binary system)					
Low/high	35/27	4/13	6/8	2/3	0/2
Tobacco use[1]	Intermediate	Heavy	Heavy	None	None
Alcohol use[2]	Light	Heavy	Heavy	None	Light

[1]Heavy smoker > 20 cigarettes/day, intermediate smoker 10–20 cigarettes/day, and light smoker < 10 cigarettes/day.
[2]Heavy drinker > 28 units/wk, intermediate drinker 15–28 units/wk, and light drinker < 14 units/wk.

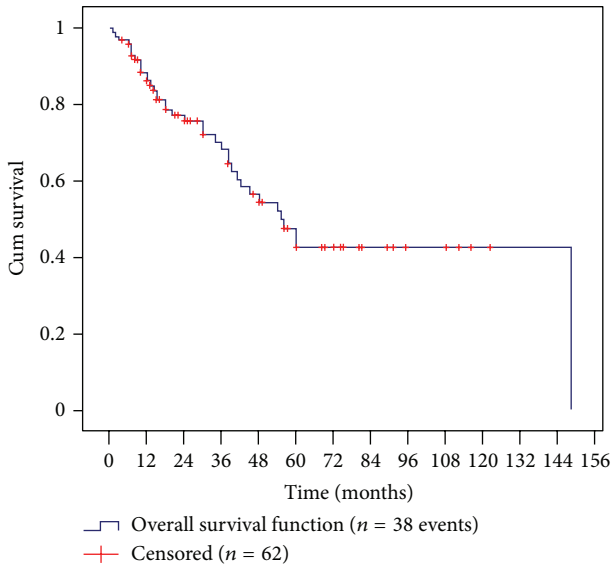

Figure 3: Kaplan-Meier analysis plotting overall disease-free survival.

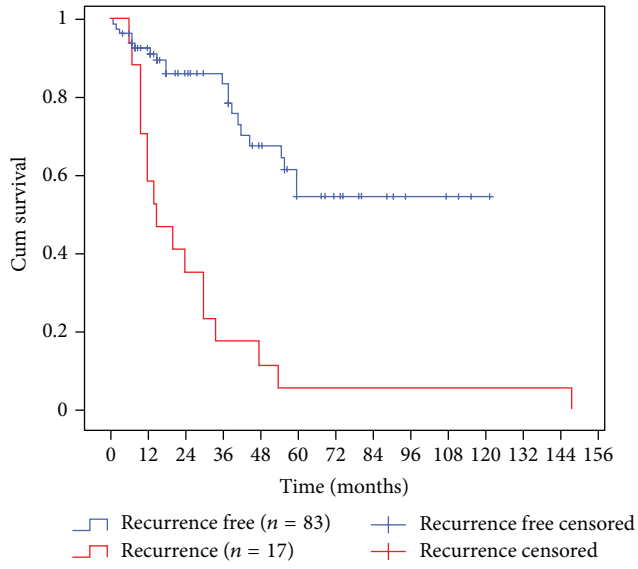

Figure 4: Kaplan-Meier analysis plotting disease-free survival for recurrent (same site) disease and recurrence-free patients (P = 0.0001, Log-Rank test).

to mild dysplasia. High-grade dysplasia was also a significant predictor for disease active state (P = 0.020), increasing the risk to approximately 3 times that of low-grade dysplasia, Table 3.

The presence of dysplasia in surgical resection margins was also a significant prognostic factor for disease active status (P = 0.035), increasing risk by nearly 3 times compared with clear margins. Major sized lesions displayed a 4.5 times increased risk compared to minor sized ones (P = 0.045), Table 3.

4. Discussion

The ability to predict clinical outcome for PMDs remains elusive in clinical practice, probably due to lack of understanding of the natural history of the disease, confusion over terminology, limited agreement on therapeutic interventions, and uncertainty regarding patient followup. This paper is unique in presenting detailed, long-term clinical outcome data for a 100 patient cohort presenting with dysplasia-proven PMDs, all excised by laser to a standardised treatment protocol. We also define clinical outcome categories and identify predictive clinico-pathological features. It is notable upon reviewing the literature that many previous authors have not found PMD clinical appearance, anatomical site, histopathological assessment, or features related to patient age, gender, or risk factor behaviour to reliably predict clinical outcome [1, 6–10].

The ultimate goal of PMD diagnosis and management must, of course, is the prevention of SCC. Malignant transformation rates varying widely between 0.1 and 40% have

Table 3: Logistic regression models for "disease active" status.

Outcome	Risk factors	Univariate analysis		Multivariable analysis	
		Odds (95% CI)	*P* value	Odds (95% CI)	*P* value
Disease active: recurrent or further dysplasia, malignant transformation, and OSCC development	Age	1.007 (0.976–1.040)	0.646		
	Sex				
	Females	Reference category			
	Males	1.448 (0.806–3.455)	0.405		
	Leukoplakia types				
	Homogenous	Reference category			
	Nonhomogenous	**2.991 (1.160–7.713)**	**0.023**	3.319 (0.799–13.779)	0.099
	PMDs site				
	FOM	Reference category			
	Tongue	**3.381 (1.292–8.845)**	**0.013**	3.323 (0.775–14.241)	0.106
	Other remaining sites	**2.893 (0.971–8.620)**	**0.057**	0.944 (0.171–5.218)	0.947
	Histopathology (WHO grading)				
	Mid dysphasia	Reference category			
	Moderate	1.129 (0.350–3.641)	0.839	1.960 (0.419–9.167)	0.393
	Severe	**4.622 (1.527–13.990)**	**0.007**	**5.994 (1.282–28.018)**	**0.023**
	CIS	**4.800 (1.123–20.479)**	**0.034**	**17.104 (2.427–120.561)**	**0.004**
	Binary grading				
	Low grade	Reference category			
	High grade	**2.828 (1.182–6.678)**	**0.020**		
	Resection margin				
	Free margins	Reference category			
	Dysplastic margins	**2.812 (1.073–7.371)**	**0.035**	**6.562 (1.545–27.878)**	**0.011**
	PMDs size (mm^2)				
	Minor < 200	Reference category			
	Intermediate 200–600	2.327 (0.944–5.740)	0.067		
	Major > 600	**4.464 (1.035–18.394)**	**0.045**		

been quoted in the literature, which is extremely unhelpful in individual patient management, although the highest risk of cancer development is believed to occur in the most dysplastic precursor lesions; larger mucosal lesions and nonsmoking patients also appear to be at enhanced risk of malignancy [7, 8, 11, 12].

A number of observational, anecdotal, and retrospective papers have reported clinical outcome and malignant transformation data through the years, but these are significantly weakened by the heterogeneous clinical and histopathological nature of the precursor lesions studied and by a lack of any agreed treatment protocols and uncoordinated follow-up regimes. Table 4 lists the malignant transformation rates seen in the dysplastic lesions reported in these studies; none of the studies distinguished between same site and new site cancer, but overall malignant transformation rates in excess of 36% (with a mean of 16%) were reported, which are significantly higher than either the 2 new site SCC cases or the 5 same site malignant transformations seen during this study [9, 13–20].

More recently, Mehanna et al. quoted a 12% cancer rate over a mean transformation time of 4.3 years using a systematic review and meta-analysis which included a total of 992 cases although interestingly, because of limitations in the published literature, they only felt able to include 14 papers out of a possible 2837 identified oral precancer publications. The authors reported a lack of high quality evidence to date which limited the scope of their study [21].

To date, there has been a paucity of prospective, randomised controlled trials in oral precancer research, and those that do exist have not fundamentally resolved treatment and clinical outcome dilemmas. This 100 patient cohort study, although not a controlled trial population, is a unique data set facilitating analysis of a defined oral precancer population with shared risk factor behaviour presenting with proven dysplastic PMDs, standardised diagnostic and treatment protocols, consistent clinical decision making, and longitudinal patient observation with documented clinical followup.

Whilst interventional laser excision of mucosal dysplastic lesions appears to reduce the risk of same site malignant transformation, SCC development at new sites remains a risk reflecting field change in PMD disease [2, 3, 10]. Active mucosal surveillance and regular clinic follow up remain mandatory for all PMD patients, and interventional management strategies are best regarded as cyclical, passing from active surgical excision through to surveillance and then returning to surgical intervention for early targeting of

Table 4: Malignant transformation of dysplastic precursor lesions.

	Number of dysplastic lesions	Study period (yrs)	Malignant transformation (%)
Silverman et al. (1984) [9]	22	7	36.4
Hogewind et al. (1989) [13]	84	5	3.6
Lumerman et al. (1995) [14]	44	3	16
Schepman et al. (1998) [15]	166	24	12
Cowan et al. (2001) [16]	165	20	14
Holmstrup et al. (2006) [17]	87	20	12
Hsue et al. (2007) [18]	166	10	4.8
Ho et al. (2009) [19]	33	10	24
Arduino et al. (2009) [20]	207	16	7.2
Liu et al. (2011) [12]	138	5	26.8
Warnakulasuriya et al. (2011) [8]	204	9	11.7
Ho et al. (2012) [11]	91	5	25.3
Brouns et al. (2013) [7]	56	4	14.3

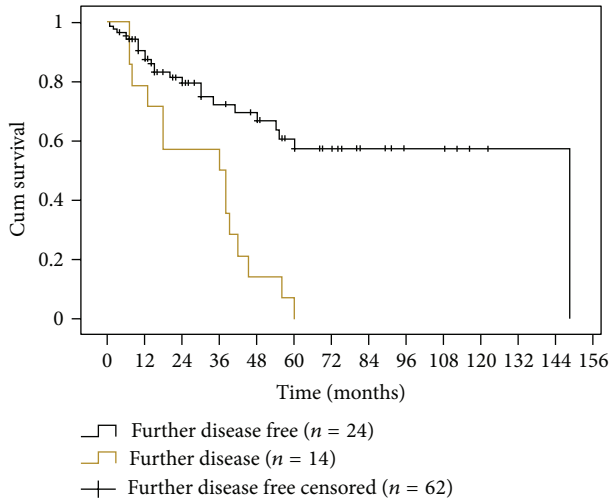

Figure 5: Kaplan-Meier analysis plotting disease-free survival for further (new site) disease and further disease-free patients (P = 0.0001, Log-Rank test).

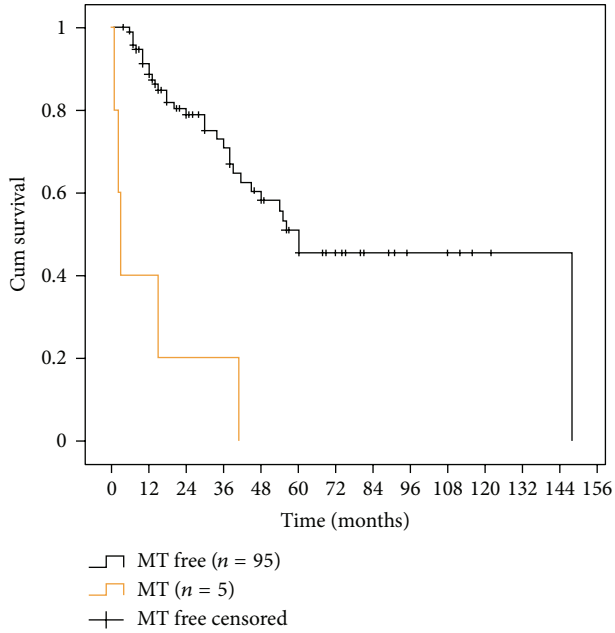

Figure 6: Kaplan-Meier analysis plotting disease-free survival for (same site) malignant transformation and malignant transformation-free patients (P = 0.0001, Log-Rank test).

further PMD disease. A particular advantage of laser surgery is the ability to repeat excisions or ablations at the same site on a number of occasions, without compromising oral healing or function [2, 10].

5. Conclusions

In this study, 62% of PMD patients were disease-free following laser excision of dysplastic mucosal lesions, and the risk of malignant transformation remained low at 2 to 5%. The incidence of further disease, however, increased with the length of patient followup, and was higher for non-homogeneous leukoplakias, large mucosal lesions, more severe dysplasias, floor of mouth and ventral tongue sites, and in nonsmoking patients. Long-term patient followup and active clinical surveillance thus remains mandatory for all PMD patients. Multicentre, randomised controlled clinical trials for PMD treatment are now urgently required to determine treatment efficacy.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Human Calmodulin-Like Protein CALML3: A Novel Marker for Normal Oral Squamous Mucosa That Is Downregulated in Malignant Transformation

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Oral cancer is often diagnosed only at advanced stages due to a lack of reliable disease markers. The purpose of this study was to determine if the epithelial-specific human calmodulin-like protein (CALML3) could be used as marker for the various phases of oral tumor progression. Immunohistochemical analysis using an affinity-purified CALML3 antibody was performed on biopsy-confirmed oral tissue samples representing these phases. A total of 90 tissue specimens were derived from 52 patients. Each specimen was analyzed in the superficial and basal mucosal cell layers for overall staining and staining of cellular subcompartments. CALML3 was strongly expressed in benign oral mucosal cells with downregulation of expression as squamous cells progress to invasive carcinoma. Based on the Cochran-Armitage test for trend, expression in the nucleus and at the cytoplasmic membrane significantly decreased with increasing disease severity. Chi-square test showed that benign tissue specimens had significantly more expression compared to dysplasia/CIS and invasive specimens. Dysplasia/CIS tissue had significantly more expression than invasive tissue. We conclude that CALML3 is expressed in benign oral mucosal cells with a statistically significant trend in downregulation as tumorigenesis occurs. CALML3 may thus be a sensitive new marker for oral cancer screening.

1. Introduction

Approximately 30,000 new cases of cancer in the oral cavity and oropharynx are diagnosed in the United States each year, corresponding to about 3% of all malignant tumors. Although visibly detectable due to the accessibility of the oral cavity, oral cancer has a high morbidity and mortality because it is typically at an advanced stage when it is finally clinically visible. Accordingly, the five-year survival rate for all stages combined is only 51%. Of the cancers involving the oral cavity and oropharynx, more than 90% are squamous cell carcinomas [1–3]. Up to 40% of those diagnosed with squamous cell carcinoma will develop a metastatic tumor or a second primary tumor to a nearby organ at a later time.

Early diagnosis of oral and oropharyngeal squamous cell carcinoma is crucial for a more favorable prognosis. The oral cavity and oropharynx are easily accessible for routine screening for squamous cell carcinoma, but reliable detection in early stages will require robust markers that are easy to assay. Methods for testing oral epithelial cells for malignancy traditionally involve an invasive and often painful biopsy. Whether as a diagnostic tool or prognostic marker, the potential to develop a simple, less invasive method for oral cancer screening would be of great value.

Human calmodulin-like protein CALML3 (synonyms: CLP, calmodulin-related protein NB-1) is a 148-amino-acid-residue calcium sensor protein closely related to the ubiquitous calmodulin [4, 5]. However, in contrast to calmodulin, CALML3 is tissue specific and seems to be expressed almost exclusively in normally differentiating epithelia such as those of breast, thyroid, prostate, kidney, and skin [6]. In a study comparing normal reduction mammoplasty specimens to archival breast cancer specimens, Rogers et al. [7] found that CALML3 was expressed at high levels in normal breast epithelium and that significant CALML3 downregulation occurred in 79% to 88% of invasive ductal carcinoma and lobular carcinoma specimens. The authors concluded that CALML3 downregulation is common in breast tumorigenesis and that the status of CALML3 expression can serve as a marker for the nonmalignant state [7].

We have previously found that CALML3 is expressed at the mRNA transcript level and can be detected by immunohistochemistry in normal oral mucosa tissue. By contrast, there was a notable reduction in immuno staining in areas of malignant transformation [8]. Here we performed immunohistochemical analysis to compare CALML3 expression in tissue samples representing the various phases of oral tumor progression to see if there is a statistically significant trend of changes in CALML3 expression during disease progression. In addition, direct comparisons in CALML3 expression were made between the individual categories of benign, dysplasia, carcinoma in situ, and invasive squamous cell carcinoma.

2. Materials and Methods

2.1. Tissue Specimens. Under IRB-approved protocol 754-04, the Mayo Clinic Tissue Registry was searched for patients that had undergone surgical biopsy of the oral cavity. A database was compiled placing the tissue specimens into 4 categories: benign squamous mucosa (including mild reactivity and hyperplasia), squamous dysplasia, squamous cell carcinoma in-situ (CIS), and invasive squamous cell carcinoma. The invasive squamous cell carcinoma was further categorized by grade [9]. The database was cross-referenced for patients who had given research consent. H&E stained slides for the surgical procedures were evaluated and the most appropriate tissue block for each case was processed for CALML3 immunostaining. A staff surgical pathologist (Thomas J. Sebo) confirmed the diagnosis of each sample. Samples were eliminated if there were any questionable diagnoses or if the quality of the section was poor.

A total of 90 tissue specimens were derived from 52 patients with 62 surgical procedures. For the purpose of this study, the assumption was made that multiple specimens from the same patient are independent. Specimens from the same patient were generally from different lesion sites taken during more than one date of surgery. Among the 52 patients, 31 (60%) were male and the overall mean age at the time of diagnosis was 64 years (SD, 15; median, 67; range, 6 months–87 years). Among the 90 tissue specimens, 30 (33%) were diagnosed as benign, 6 (7%) as dysplastic, 12 (13%) as carcinoma in situ, and 42 (47%) as invasive squamous cell carcinoma. Of the 42 samples diagnosed as squamous cell carcinoma, 2 (5%) were grade 1, 11 (26%) were grade 2, 27 (64%) were grade 3, and 2 (5%) were grade 4. Other data recorded included location of specimen (tongue, floor of mouth, right, left, etc.) and type of surgery (excision versus biopsy).

2.2. Immunohistochemistry. The generation, affinity purification, and characterization of a rabbit polyclonal antibody (TG7) against human CALML3 have been described [7]. These antibodies (generated by Cocalico, Inc., Reamstown) recognize a peptide corresponding to the C-terminal residues 127 to 148 of CALML3 (the most divergent region between CALML3 and calmodulin). Affinity-purified antibodies showed excellent sensitivity and specificity for CALML3 [5, 7]. The paraffin-embedded tissue specimens were cut at a thickness of 6 μm, mounted on positively charged slides, deparaffinized, and treated with H_2O_2/methanol to block endogenous peroxidase activity essentially as described [10, 11]. The specimens received heat-induced epitope retrieval in 1 mM EDTA at a pH of 8.0 for 20 minutes. Nonspecific protein-binding sites were blocked in 5% normal goat serum in phosphate-buffered saline solution (PBS)/0.05% Tween-20 for 1 h, and the sections were immunostained by sequential incubations (1-2 h at room temperature each) in affinity-purified CALML3 antibody TG7 (40 μg/mL in PBS/0.05% Tween-20/1% normal goat serum), biotinylated goat anti-rabbit IgG (1 : 200 in PBS/0.05% Tween-20/1% normal goat serum, DAKO, Carpinteria, CA), and horseradish peroxidase-conjugated streptavidin (1 : 300, DAKO, Carpinteria) essentially as described [7]. Slides were rinsed 3 × 5 min in PBS/0.05% Tween-20 between incubations. The sections were then incubated in 3-amino-9-ethylcarbazole in the presence of H_2O_2 and counterstained with hematoxylin and mounted with coverslips.

Two observers (a staff surgical pathologist (Thomas J. Sebo) and a maxillofacial prosthodontist (Michael D. Brooks)) evaluated the specimens for expression of CALML3. Staining patterns and intensity were observed and recorded for all categories of samples (benign, dysplasia/carcinoma in-situ, and invasive squamous cell carcinoma). All slides were evaluated at the same magnification. For each specimen, 3–5 separate fields of view were analyzed in 2 mucosal cell layers: superficial cells and basal cells. Analysis of staining in cellular compartments was further subdivided into cytoplasmic membrane (CM), cytoplasm (C), and nucleus (N). Staining was graded on a 4-point scale: 0: absent (no staining above background of a negative control incubated with anti-CALML3 IgG preabsorbed with CALML3 [6]), 1: weak (barely above background and no strong staining in specific cellular regions), 2: intermediate (moderate staining with varying degree of subcellular staining), 3: strong (intense staining with distinct cellular localization). The cytoplasmic membrane, cytoplasm, and nuclei for each mucosal cell region were individually assigned a grade.

Assigning a grade to each specimen type followed a few basic and logical rules. First, the squamous cells were initially evaluated using two low magnification lenses (2.5x and 5.0x for a total magnification strength of 25x and 50x) to obtain a general sense of staining intensity of all the squamous cells on

the slide. Second, for cytoplasmic membrane staining, cells either exhibited circumferential membrane staining or no membrane staining. Third, membrane staining, apart from cytoplasmic staining, was typically most readily apparent in the intermediate layer of the squamous mucosal cells. As the cells reach the actual superficial layer of, for example, benign mucosa and become flattened, the ability to distinguish cytoplasmic staining from cytoplasmic membrane staining was limited.

Invariably, tissue samples in which no CALML3 staining in the squamous cells was detected could very quickly be assigned a grade of 0 (no staining). In the situation of superficial squamous cell staining, this occurred in 25%–50% of the case material. Conversely, strong (3+) CALML3 immunostaining could be readily ascertained using the same approach in 5%–30% of the case material. In evaluating basally positioned squamous cells, no staining could be detected very quickly in 50% to almost 100% of the case material, whereas strong (3+) staining was very rare (about 2%).

The challenge in this study, and all studies in which immunostain intensity is visually graded, came in grading squamous cells as displaying either weak (1+) or intermediate (2+) CALML3 expression. In these instances, an overall assessment of staining intensity at low magnification showed that the degree of staining could not clearly be categorized as 0 or 3+, and a close analysis of all squamous cells in the tissue sample was performed using all magnification lenses (2.5x, 5x, 10x, 20x, and 40x for overall magnification of 25x, 50x, 100x, 200x, and 400x) to arrive at a grade reflecting the staining intensity for all squamous cells. Thus, a grade of 1+ (weak) indicates that the overall expression of the squamous cells was weak, showing only barely visible staining above 0. For an intermediate grade (2+), the degree of CALML3 staining was felt to be unquestionably present but not as visually strong as a grade of 3+.

On occasion, rare cells within any given tissue specimen graded as 1+ or 2+ showed strong (3+) or no (0) expression. However, no effort was made to further stratify case material on the basis of the percentage of cells showing a range of CALML3 expressions. As such, our grading system reflects an overall score of CALML3 staining intensity. In those instances in which the two reviewers (Thomas J. Sebo and Michael D. Brooks) recorded different grades for a given case, rereview of the case material was undertaken to arrive at a consensus grade. This occurred in roughly 10% of the case material.

2.3. Statistical Methods. For analysis purposes, grades 0 and 1 were collapsed and reported together as no/weak staining and grades 2 and 3 were collapsed and reported together as intermediate/strong stain. This is because visually weak staining most closely approximates no (0) staining and intermediate (2+) staining most closely approximates strong (3+) staining. Total stain scores were derived by summing the stain grades for the three cellular compartments. The Cochran-Armitage test for trend was used to evaluate whether the proportion of specimens with intermediate/strong expression significantly changed with increasing disease severity. In addition, the chi-square test (or the Fisher's exact test, as appropriate) was used to compare the proportion of specimens with intermediate/strong expression between the benign, dysplasia/CIS, and invasive tissue groups. The Kruskal-Wallis test was used to compare the total stain scores between the three groups. If the P value for the overall test of group differences was <0.05, then pair-wise comparisons among the three groups were performed. The analyses assumed that multiple specimens from the same patient were independent. All calculated P values were two-sided and P values less than 0.05 were considered statistically significant. A Bonferroni correction could be applied by assessing the pair-wise comparisons between the three tissue types using an alpha level of 0.0167 to account for the three comparisons. Statistical analysis was performed using the SAS software package (SAS Institute, Cary, NC).

3. Results

As we expected from previous studies [6, 8], benign mucosa displayed robust immunostaining (Figures 1(a) and 1(b)). Distinct staining was noted in the periphery (cytoplasmic membrane) and nuclei of the more superficially located epithelial cells (Figure 1(b)). On the other hand, in samples with dysplasia (Figures 1(c) and 1(d)) and carcinoma in-situ (not shown), a marked decrease was seen in strength of stain. A strong reduction (or complete lack) of CALML3 immunostaining was seen in invasive squamous cell carcinoma compared to both benign tissue and dysplasia/CIS tissue (Figures 2 and 3), although in low-grade invasive squamous cell carcinoma, keratin pearls did illustrate mild immunoreactivity. The basal cell layers in general did not stain as intensely as the more superficial cell layers, and no statistical difference was seen with basal nuclear and cytoplasmic staining among the groups.

Table 1 summarizes the CALML3 expression results at the superficial and basal mucosa levels, separately for the tissue types. Because similar results were found for the dysplastic and CIS specimens, these two tissue types were combined for the analysis. In addition, we found that aside from the superficial cytoplasmic membranes and nuclei of the benign specimens, only a small percentage of the other tissue regions exhibited strong expression. Therefore to evaluate whether there was a trend in the expression level with tissue disease severity, the specimens with weak or no staining were combined, as were those with intermediate or strong staining (see Table 1). However, the detailed summary of all staining results broken down by individual tissue type is available upon request.

Based on the Cochran-Armitage test for trend, the CALML3 expression level significantly decreased with increasing disease severity for the following sites: superficial cytoplasmic membrane ($P < 0.001$), superficial nucleus ($P < 0.001$), and basal cytoplasmic membrane ($P = 0.009$). On the other hand, a statistically significant trend was not observed for superficial cytoplasm ($P = 0.19$) and basal cytoplasm ($P = 0.19$), in which several dysplastic/CIS specimens had stronger expression than the benign tissue. None of the basal nucleus sites had a stronger than "weak" staining score for any of the tissue types.

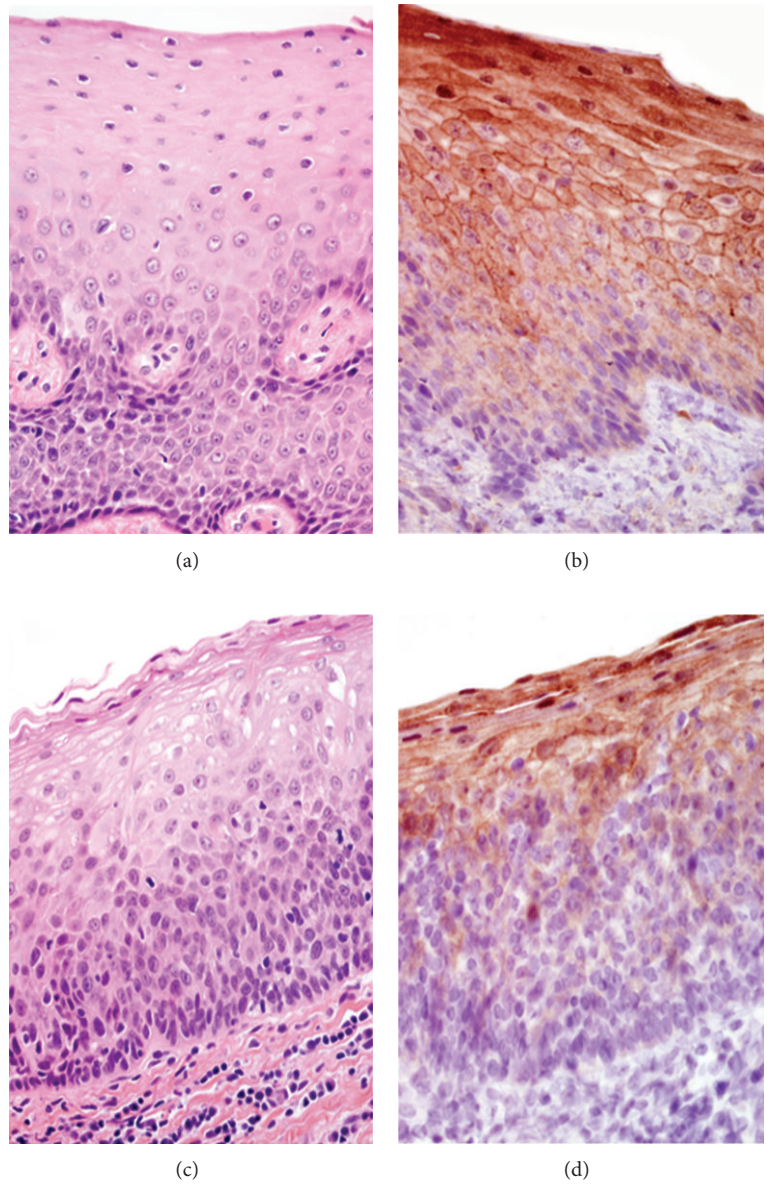
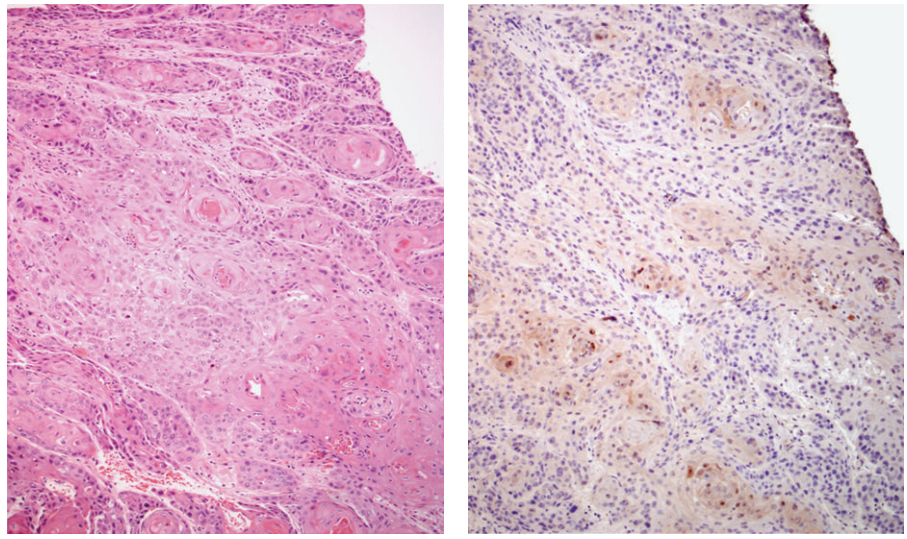


Figure 1: H&E (a) and CALML3 immunostained (b) sections of benign oral epithelium and H&E (c) and CALML3 immunostained (d) sections of dysplastic oral mucosa under high power light microscopy (200x). Immunostaining is more intense towards the superficial layers with an apparent lack of CALML3 expression from the more basaloid cells. Dense cytoplasmic membrane staining and nuclear staining can be appreciated.

The chi-square test (or the Fisher's exact test, where appropriate) was used to compare the proportion of specimens with an intermediate or strong stain between the benign, dysplasia/CIS, and invasive tissue groups (Table 1). Benign tissue specimens had significantly more CALML3 expression in the superficial mucosa compared to invasive specimens ($P < 0.001$) and dysplasia/CIS specimens ($P = 0.013$), as revealed by a comparison of the total stain scores (Table 1 and Figure 4). This difference in CALML3 staining was most pronounced for the cytoplasmic membrane and the nucleus. Dysplasia/CIS tissue also had significantly ($P = 0.003$) more expression than invasive tissue in the superficial mucosa (Table 1 and Figure 4), and this difference was most clearly demonstrated in the cytoplasm (50% versus 7.1%, $P < 0.001$) and in the nuclei (38.9% versus 2.4%, $P < 0.001$). All of the previously outlined comparisons of expression in the superficial layer between the three tissue groups retained statistical significance if a Bonferroni correction was applied and significance is assessed using an alpha level of 0.0167 instead of 0.05.

Expression was not strong in any of the sites in the basal layer (Table 1), though there was evidence suggesting a slight increase in expression in the cytoplasmic membrane of benign squamous cells compared to invasive carcinoma



(a) (b)

Figure 2: H&E (a) and CALML3 immunostained (b) sections of invasive squamous cell carcinoma under low power light microscopy (100x). An appreciably diminished, and in many areas, absent immunoreactivity is seen in invasive squamous cell carcinoma.

Table 1: Summary of staining results, by tissue type.

Cellular compartment	Tissue type			P value‡			
	Benign (N = 30)	Dysplasia/CIS (N = 18)	Invasive (N = 42)	Any of the 3 tissue types	Benign versus Dys/CIS	Benign versus invasive	Dys/CIS versus invasive
Superficial: cytoplasmic membrane				<0.001	<0.001	<0.001	0.99
No/weak stain	5 (16.7%)	14 (77.8%)	32 (76.2%)				
Intermediate/strong stain	25 (83.3%)	4 (22.2%)	10 (23.8%)				
Superficial: cytoplasm				<0.001	0.014	0.26	<0.001
No/weak stain	25 (83.3%)	9 (50%)	39 (92.9%)				
Intermediate/strong stain	5 (16.7%)	9 (50%)	3 (7.1%)				
Superficial: nucleus				<0.001	0.23	<0.001	<0.001
No/weak stain	13 (43.3%)	11 (61.1%)	41 (97.6%)				
Intermediate/strong stain	17 (56.7%)	7 (38.9%)	1 (2.4%)				
Superficial: total stain score†							
Median (IQR)	5 (5, 6)	4 (2, 5)	2 (1, 3)	<0.001	0.013	<0.001	0.003
Basal: cytoplasmic membrane				0.022	0.28	0.027	—
No/weak stain	26 (86.7%)	18 (100%)	42 (100%)				
Intermediate/strong stain	4 (13.3%)	0 (0%)	0 (0%)				
Basal: cytoplasm				0.13	—	—	—
No/weak stain	27 (90%)	15 (83.3%)	41 (97.6%)				
Intermediate/strong stain	3 (10%)	3 (16.7%)	1 (2.4%)				
Basal: nucleus				0.99	—	—	—
No/weak stain	30 (100%)	18 (100%)	42 (100%)				
Intermediate/strong stain	0	0	0				
Basal: total stain score†							
Median (IQR)	1 (0, 2)	1 (0, 2)	0 (0, 1)	<0.001	0.55	<0.001	0.010

IQR: interquartile range. Values are reported as N (%) unless otherwise noted.

†Total stain score was derived as the sum of the staining grades for the three components: cytoplasmic membrane, cytoplasm, and nucleus components. Each component was graded on a scale of 0 = absent, 1 = weak, 2 = intermediate, and 3 = strong.

‡Comparisons between the three tissues types were evaluated based on the chi-square test or Fisher's exact test for the categorical variables and the Kruskal-Wallis test for the total stain scores. If the P value for the overall test for differences was <0.05, then pair-wise comparisons among the three tissue types were performed.

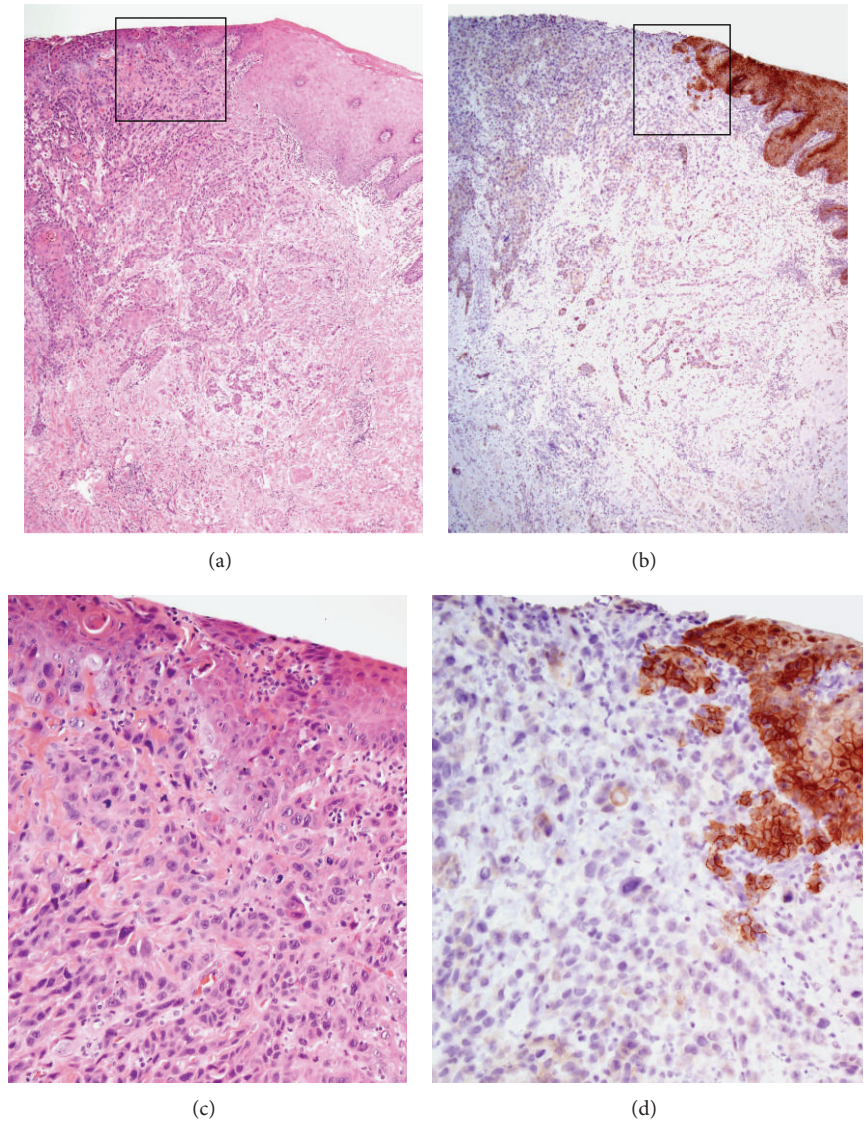


Figure 3: H&E (a) and CALML3 immunostained (b) sections of invasive squamous cell carcinoma with focal area of benign squamous mucosa under low power light microscopy (40x) and H&E (c) and CALML3 immunostained (d) sections of invasive squamous cell carcinoma with focal area of benign squamous mucosa under high power light microscopy (200x). Areas of invasive squamous cell carcinoma show a marked decrease in immunoreactivity ((b) and (d), left side of panels). Areas of intact benign oral mucosa maintain CALML3 expression with notable immunoreactivity (upper right in (c) and (d)).

(13.3% versus 0%, $P = 0.027$). In analyzing the 42 specimens of squamous cell carcinoma separately by grade, no obvious trends were apparent.

4. Discussion

Previously, loss of CALML3 immunoreactivity has been linked to early breast cancer development [7]. Here, we show that while CALML3 is strongly expressed in the superficial layers of benign oral mucosa, different oral mucosa tissue types representing the various stages of carcinogenesis exhibit a reduction of CALML3 expression as squamous cells progress from benign, to dysplastic, to carcinoma in situ, to invasive squamous cell carcinoma.

In general, a trend is seen that as disease severity increases, CALML3 expression decreases. The more differentiated the cells, the more intense the staining. Many of the benign specimens showed an increase in staining intensity the further the cells moved away from the basal cell layer. The cells in the basal layer are less mature. As the cells mature, they differentiate and migrate to the upper layers as underlying cells develop. The more intense CALML3 staining towards the outer layers of mucosa is consistent with previous reports that noted markedly increased staining in more differentiated layers of stratified epithelia [6]. Therefore, CALML3 may play a role in terminal differentiation of oral keratinocytes, as has been proposed for the basal and suprabasal keratinocytes of the epidermis during wound healing [12]. CALML3 is

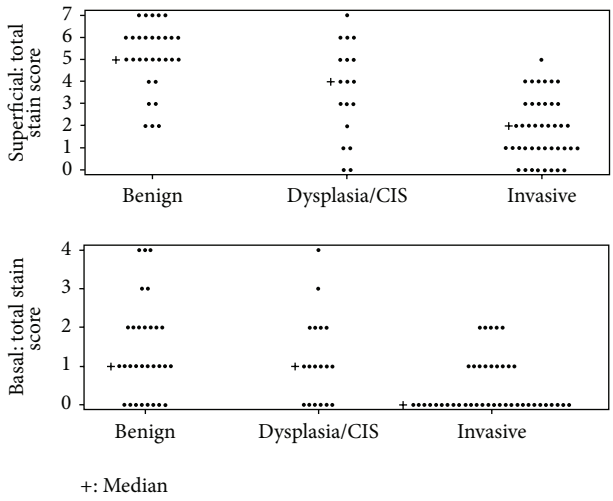


Figure 4: Total stain scores in the superficial cellular compartments and the basal cellular compartments among the tissue categories of benign, dysplasia/carcinoma in situ, and invasive squamous cell carcinoma. A statistically significant trend is seen in CALML3 expression from benign mucosal cells to invasive squamous cell carcinoma. As disease severity increases, CALML3 expression decreases.

a calcium-sensor protein related to calmodulin and as such is thought to exert its function by regulating specific target proteins. Among these, the unconventional myosin-X [13] is known to be involved in directional cell migration and cell adhesion, both are important events during terminal keratinocyte differentiation. The staining for CALML3 in the cytoplasmic membrane of superficial cells in benign oral mucosa may thus reflect its function in regulating MyoX concentrated at the cell periphery [14]. Like calmodulin, CALML3 has multiple targets, one of which (IQ motif containing protein E or IQCE) was recently identified in a yeast two-hybrid screen and may be a protein involved in DNA metabolism (Richard D. Bennett and Emanuel E. Strehler, unpublished). Such a putative nuclear CALML3 target protein(s) could help explain the nuclear staining for CALML3 that we observed in the superficial cells of the normal oral mucosa. However, additional studies are needed to determine the role of CALML3 in the nucleus and its relevance to normal epithelial differentiation.

Only few diagnostic tests are currently available for oral cancer. Techniques being implemented for diagnostic tests include transepithelial brush biopsy/exfoliative cytology, fine needle aspiration, and toluidine blue [15–23]. These techniques all rely on cytological markers to indicate the presence of malignant or premalignant cells. Although these tests are preferred over conventional biopsy confirmation because they are less invasive and more tolerable for the patient, their reliability and efficacy have not been validated [24–26]. Because the specific expression and cellular localization of CALML3 indicate normal cell function, the potential for development of a simple diagnostic test for oral cancer using CALML3 as a marker is promising. Currently diagnostic screening tools are being utilized to visualize autofluorescence in normal oral tissue with loss of autofluorescence indicative of tissue change or abnormality. Noninvasive screening could be implemented by the use of exfoliative brushing cytology, minibiopsy, or a "swish-and-spit" procedure. This would allow a clinician who observes a suspicious area in the mouth to routinely test for CALML3 levels. A change in CALML3 expression could indicate the presence or the early developing stage of oral cancer.

Authors' Contribution

Michael D. Brooks and Richard D. Bennett conceived and carried out experiments. Michael D. Brooks, Richard D. Bennett, Thomas J. Sebo, and Emanuel E. Strehler conceived experiments and analysed data. Amy L. Weaver provided statistical analysis. Steven E. Eckert and Alan B. Carr provided consultation for experiments. All authors were involved in writing the paper and had final approval of the submitted and published version.

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Research Article

Effectiveness of Fluoride Varnish Application as Cariostatic and Desensitizing Agent in Irradiated Head and Neck Cancer Patients

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Objective. To evaluate the effectiveness of three-month fluoride varnish application on radiation caries and dental sensitivity and to assess compliance to three-month fluoride varnish application. *Materials and Methods*. 190 irradiated head and neck cancer patients were randomly selected and reviewed retrospectively. Oral prophylaxis, fluoride varnish application, and treatment of dental caries were done prior to radiation therapy. Patients were followed up at every three months for dental evaluation and fluoride varnish application. Decayed-missing-filling-teeth indices, dental sensitivity, and compliance to fluoride varnish application were noted for fifteen months and analyzed statistically. *Results*. Significant increase in decayed-missing-filling-teeth index was seen at nine ($P = 0.028$), twelve ($P = 0.003$) and fifteen ($P = 0.002$) months follow-up. However, the rate of increase in decayed-missing-filling-teeth indices was 1.64/month which is less than the rate mentioned in the literature (2.5/month). There was no significant effect of sex ($P = 0.952$) and surgery ($P = 0.672$) on radiation caries, but site of disease ($P = 0.038$) and radiation dose ($P = 0.015$) were found to have statistically significant effect. Dental sensitivity decreased from 39% at 3 months to 25% at 15 months followup. 99% compliance to fluoride varnish application was seen till six months followup which decreased to 46% at fifteen months. *Conclusion*. Three-month fluoride varnish application is effective in decreasing radiation caries and sensitivity and has good compliance.

1. Introduction

Radiation therapy plays an important role in the management of patients with head and neck cancer. It is also associated with several undesired reactions. In the long term, the irradiated patients are susceptible to atrophy and fibrosis of the muscles of mastication that can lead to trismus and xerostomia leading to extensive dental caries and osteoradionecrosis [1].

Radiation caries is a specific form of dental caries with multifactorial etiology. It is highly destructive with a rapid onset, progression, and nonspecific localization. Xerostomia is the main risk factor [2]. An ideal approach to prevent radiation caries is quantitative and qualitative modification of saliva [1]. Radiation-induced hyposalivation can be avoided by excluding major and minor salivary glands from irradiation field [3].

Topically applied fluorides buffers pH of saliva reduces oral cariogenic flora and remineralises tooth structure there by qualitatively altering the saliva [4]. As desensitizing agents, fluorides work by blocking the dentinal tubules and prevent the movement of fluid backward and forward within the dentinal tubules in response to stimuli of pain [5].

Radiated head and neck cancer patients are high risk patients for dental caries and dental sensitivity. As per American Dental Association (ADA) recommendations for high risk patients, the fluoride varnish should be applied 2–4 times per year. The fluoride varnish has been found to be effective in preventing caries in high risk patients [6].

The successful use of topically applied fluorides to prevent radiation caries has been described by several authors. Daly et al. [7] and Dreizen et al. [8] reported the use of 1.0% neutral NaF, or NaF_2 gel (4,500 parts per million fluoride ion), applied daily in custom tray. However, the compliance with fluoride application in carriers by the population of patients with head and neck cancer is generally thought to be poor [9, 10].

The aim of this research was to determine the effect of fluoride varnish application on radiation caries and dental sensitivity in head and neck cancer patients. The intention was also to assess the compliance of patients to three monthly fluoride varnish application (FVA).

2. Materials and Methods

Head and neck cancers are one of the most prevalent cancers in India in view of the social habits. Almost all of these patients receive definitive or adjuvant radiation therapy as part of the treatment.

A complete clinical and radiological oral examination is considered as an integral component of overall medical care at our institute before initiation of radiation therapy. Existing decayed teeth are salvaged, if possible, or extracted. Thorough oral prophylaxis, followed by application of slow release aqueous-based topical 5% NaF varnish (Fluoritop-SR, ICPA Health Products Ltd. Mumbai, India), is done. Detailed oral hygiene instructions are given to the patients. These patients are followed up every three months for dental evaluation and FVA by single clinician.

190 head and neck cancer patients, who received radiation therapy, were randomly selected and reviewed. These patients had completed clinical and radiological oral examinations before initiation of radiation therapy. All these patients had undergone thorough oral prophylaxis, and FVA was done prior to radiation therapy and at every three months follow-up. Preradiotherapy, followup decayed-missing-filled-teeth Index (DMFT), dental sensitivity, and compliance to three-month FVA were recorded till fifteen months followup.

Patients' demographics, tumor location, staging, histopathology, radiation dosage, and surgery were recorded. All these patients were treated with conventional 2D radiation therapy technique. The patients were divided into three groups depending on their radiation dose, namely, Group 1: <50 Gy, Group 2: 50–60 Gy, and Group 3: >60 Gy. Statistical calculations were performed by using Mann-Whitney U test or Kruskal-Wallis test (as appropriate) for continuous variables and chi-square test or Fisher's exact test for categorical variables. All of the tests were conducted at 5% level of significance (2-sided). The data was entered in SPSS v18 software and was analyzed statistically using repeated measures ANOVA (with Bonferroni Correction). Numeric data is represented as mean ± standard deviation or frequency.

3. Results

A total of 190 patients were included in the study with 138 males and 52 females. The mean age of patients was 46.5 years (SD = 13.5, range from 16 to 77 years). The diagnosis (histopathology and site) is shown in Tables 1 and 2. The patients had been treated with 2D external beam radiation therapy to the mean dose of 58.4 Gy (SD = 8.3, range = 20–75 Gy). The majority of the patients, 71.2%, had received combination therapy of surgery and radiation therapy with or without chemotherapy. 28.2% of patients were treated with radiation therapy alone. 10 patients were treated with total dose less than 50 Gy, 95 patients received radiation dose between 50 and 60 Gy, and 36 were delivered a dose higher than 60 Gy. Radiation dose for 49 patients could not be retrieved from the records.

Table 1: Site of primary tumor (n = 190 patients).

Site of primary tumor	No.
Oral cavity	108
Oropharynx	35
Salivary glands	12
Para nasal sinus	20
Others (larynx, nasopharynx, and unknown primary)	33

Table 2: Type of primary tumor (n = 190 patients).

Type of primary tumor	No.
Squamous cell carcinoma	156
Salivary gland tumor	15
Undifferentiated carcinoma	04
Others	15

Table 3: Statistical evaluation of DMFT.

	Before RT	3 months	6 months	9 months	12 months	15 months
Mean	4.12	4.28	4.46	4.83	5.04	5.14
SD	4.35	4.42	4.85	5.15	5.31	5.36
Max.	32	32	32	32	32	32
Min.	0	0	0	0	0	0
P value		0.188	0.725	0.028	0.003	0.002

Preradiotherapy mean of initial DMFT for patients was 4.1 ± 4.3 (Table 3). With the three-month FVA, the mean DMFT increased to 5.1 ± 5.4 at fifteen months follow-up visit. The caries incremental rate was (a) preradiotherapy to six months—1.3/month (8.02%), (b) six months to fifteen months—1.7/month (15.35%), and (c) preradiotherapy to fifteen months—1.6/month (24.6%).

Statistically significant increase in DMFT index was seen at nine (P = 0.03), twelve (P = 0.003), and fifteen (P = 0.002) months indicating that the progression of radiation caries is a late effect of radiation therapy (Table 3). When the data was compared for DMFT across the study period with respect to sex of the patient or surgery as an additional treatment modality, it was seen that sex (P = 0.952) and surgery (P = 0.107) had no significant effect on radiation caries, but the site of the disease (P = 0.038), and radiation dose (P = 0.015) were found to have statistically significant effect. Due to wide variation in the number of patients for different sites and different radiation dose groups, the significance for DMFT index with the study period was not done.

Only 2.6% of patients had preradiotherapy dental sensitivity. This increased to 39% at three-month followup. Though the increase in sensitivity was highly significant (P < 0.001) at each follow-up visit when compared to base line, it was not significant when compared between consecutive follow-up visits. Significant decrease in sensitivity was seen between

twelve and fifteen months follow-up visits ($P = 0.003$). With the regular FVA, sensitivity decreased to 25% at fifteen-month followup (Figure 1).

Compliance to FVA was calculated with patient's follow-up visit. It was observed that the compliance was good till nine-month followup and gradually decreased thereafter till fifteen months (Figure 2).

4. Discussion

Preventive measures for radiation caries before, during, and after radiotherapy are necessary and should include instructions regarding a noncariogenic diet, thorough regular oral hygiene, and application of fluoride. Though consensus exists regarding fluoride application in these patients, controversy persists about type of fluoride, frequency, concentration, and method of fluoride application due to a lack of fundamental research in this field [11].

As per the author's knowledge, there are no studies in the literature where the dental caries index and effectiveness or compliance of professionally applied fluoride varnish at three-month interval are studied in Indian head and neck cancer patients.

Dreizan et al. [8] studied the incidence of radiation caries in patients with three different caries protective regimens, that is, plaque-disclosing dye: non fluoride gel, unrestricted diet, plaque-disclosing dye: fluoride gel, unrestricted diet and plaque-disclosing dye: fluoride gel, sucrose-restricted diet. They found that the mean caries incremental rate was 1.3/month in Group I. In Group II, patients who were on 1% sodium fluoride regimen with plastic gel carriers at three-month interval for first postradiation year and at six-month interval thereafter, the mean caries increment rate was 0.07/month. 0.03 mean caries incremental rate per month was seen in Group III.

In Bosnian population, Konjhodzic-Prcic et al. [2] reported caries incremental rate of 3.5/month for irradiated head and neck cancer patients. However, there was no caries protective regimen mentioned.

In our study with the three-month FVA, the caries incremental rate was found to be 1.6/month for fifteen months. However, it was seen that the rate of caries progression was slower initially, till first six months, that is, 1.3/month. The caries incremental rate increased after six months to 1.7/month. The rate of increase in radiation caries was less than that mentioned in the literature, 2.5/month [7, 12].

Due to unequal distribution of patients in different sites (Table 1) and different radiation dose group, though the site ($P = 0.038$) and radiation dose ($P = 0.015$) showed statistically significant effect on DMFT ($P = 0.038$), no inference could be obtained for site specificity, radiation dose and DMFT.

It has also been stated that during and following radiotherapy, the teeth may become hypersensitive, which could be related to the decreased secretion of saliva and the lowered pH of secreted saliva. The topical application of a fluoride relieves these symptoms [13]. Studies have found that

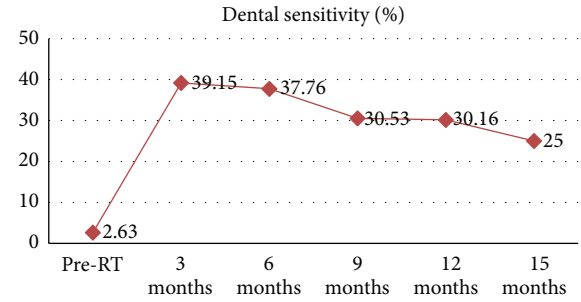

Figure 1: Graph showing dental sensitivity at each followdup visit after FVA.

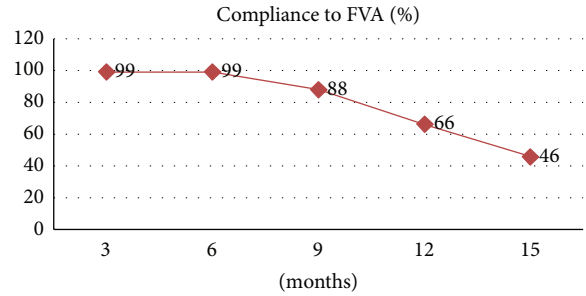

Figure 2: Graph showing compliance of the patient to FVA.

topical fluoride application forms fairly insoluble globules like calcium fluoride-like material on the tooth surface. These globules act as a reservoir of fluoride and block dentinal tubules thereby reducing dental sensitivity [14].

The retrospective analysis of the data in this study supports the documentation made in the literature regarding radiation induced dental sensitivity and fluoride treatment for the same. It was observed that the radiation sensitivity increased significantly by 36.5% at three-month followup after radiation therapy. With the regular FVA, it was seen that radiation sensitivity decreased to 25% at fifteen-month followup.

In a systemic review by Marinho [15], the assessment of effectiveness of fluoride toothpastes, gels, varnishes, and mouth rinses through comparisons against nonfluoride controls, against each other, and against different combinations is done. The average decayed-missing-filled-surface indices prevented fraction that is, percentage caries reduction ranged from 24% for fluoride toothpaste to 26% for mouth rinses and 28% for gels to 46% for fluoride varnishes.

The long-term compliance with the use of fluoride gel in custom tray has been reported as very poor by Carl [9], Boyett [16] (57% noncompliant), and Daly et al. [7] (85% fair to poor compliant). The explanation given by Jansma et al. [17] was that the use of tray was inconvenient and time consuming for patients. Shannon and Edmonds [18] considered painful mucositis following radiotherapy very discouraging to the performance of oral hygiene procedures. Joyston-Bechal et al. [19] supported the fact that the cancer patients are often depressed to concentrate on prophylactic dental procedures. Lockhart and Clark [20] reported the gagging with trays and

sociological factors leading to lack of concern for general health and hence additional cause for poor compliance.

It has been reported that the fluoride varnish is easy to apply, creates less patient discomfort, achieves greater patient acceptability, and has lesser toxicity than fluoride gel. Quantity of fluoride in varnish is less than the gel thus reduces the risk of inadvertent ingestion. However, to our knowledge, there are no articles in the literature with the assessment of compliance to three-month FVA in radiated head and neck cancer patients.

Due to lack of data in the literature and in the view of the advantages of fluoride varnish over gels, compliance to the three-month FVA protocol is assessed. It is observed that the compliance was almost 100% till six months. Poor long-term compliance of these patients can be attributed to long distance travelling, financial constraints, progression of disease, death due to disease, or other reasons. Though there was a definite decrease in compliance, it was still better than the compliance stated in the literature for fluoride gel application.

5. Limitations

This is a retrospective study, thus, there is a lack of local historical controls or concurrent controls to allow any conclusion on the impact of flouride varnish on caries in HNC patients. Xerostomia, diet frequency, sucrose intake, and so forth, which are recognized as risk factors in these patients, are not documented. Prospective studies can be conducted to assess the correlation of xerostomia, fluoride varnish application, diet, and dental caries in head and neck cancer patients.

Also, the data of noncompliant patient for DMFT index and dental sensitivity was not recorded. Thus, the potential effect of fluoride in the compliant versus noncompliant population could not be assessed, though there is a dichotomy in compliance with varnish application.

6. Conclusion

Radiation caries is the late effect of radiotherapy. Three-month FVA helps in decreasing the incidence of radiation caries. It also helps in decreasing the radiation induced dental sensitivity. Though the compliance with three-month FVA is better than fluoride gel in custom carrier, there is still a need to educate these patients about the need of FVA. Future studies with larger sample and near equal distribution of patients for specific sites and different radiation dose are required to know the effect of site specificity and radiation dose on radiation caries. Continuing studies comparing different methods of topical fluoride application in head and neck cancer patients can guide the clinician for selection of better topical fluoride.

Conflict of Interests

The authors declare that they have no conflict of interests.

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