ORAL DISEASES

Oral Diseases (2011) 17 (Suppl. 1), 7–22. doi:10.1111/j.1601-0825.2011.01789.x

www.wiley.com

ORIGINAL ARTICLE

Genetics/epigenetics of oral premalignancy: current status and future research*

MW Lingen[1], A Pinto[2,3], RA Mendes[4], R Franchini[5,6], R Czerninski[7], WM Tilakaratne[8], M Partridge[9], DE Peterson[10], S-B Woo[11]

[1]*Department of Pathology, The University of Chicago Pritzker School of Medicine, Chicago, IL, USA;* [2]*Department of Oral Medicine, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA;* [3]*Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA;* [4]*Department of Health Sciences, Portuguese Catholic University, Viseu, Portugal;* [5]*Oral Pathology and Medicine Unit, Department of Surgery and Dentistry, University of Milan, Milan, Italy;* [6]*Odontostomatology Unit, Department of Medical Science, Eastern Piedmont University, Novara, Italy;* [7]*Department of Oral Medicine, Hebrew University-Hadassah School of Dental Medicine, Jerusalem, Israel;* [8]*Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka;* [9]*Guy's and St. Thomas' Hospitals and Kings College, London, UK;* [10]*Department of Oral Health and Diagnostic Sciences, School of Dental Medicine and Neag Comprehensive Cancer Center, University of Connecticut Health Center, Farmington, CT, USA;* [11]*Division of Oral Medicine and Dentistry, Brigham and Women's Hospital, Boston, MA, USA*

Squamous cell carcinoma (SCC) of the oral and oropharyngeal region is the sixth most common malignancy in the world today. Despite numerous advances in treatment, long-term survival from this disease remains poor. Early detection can decrease both morbidity and mortality associated with this neoplasm. However, screening for potentially malignant disease is typically confounded by difficulty in discriminating between reactive/inflammatory lesions *vs* those lesions that are premalignant in nature. Furthermore, the histologic diagnosis of dysplasia can be subjective and is thus prone to a considerable range of interpretation. Similarly, no definitive, validated criteria exist for predicting which dysplastic lesions are most likely to progress to cancer over time. Given this state of science, the presence of dysplasia can only be used to indicate that an oral lesion may have an increased risk of malignant transformation. Molecular biomarkers capable of identifying the subset of lesions likely to progress to cancer are required to eliminate this clinical diagnostic dilemma. The purpose of this review is to assess the current state of knowledge regarding genetic/epigenetic alterations observed in oral mucosal premalignancy. In addition, recommendations for future research studies directed at defining the predictive capacity of specific biomarkers in this modeling are presented.

Oral Diseases (2011) **17** (Suppl. 1), 7–22

Correspondence: Sook-Bin Woo, DMD, MS, Division of Oral Medicine and Dentistry, Brigham & Women's Hospital, 1620 Tremont St., #3-028, Boston, MA 02120, USA. Tel: 617-732-6570, Fax: 617-232-8970, E-mail: swoo@rics.bwh.harvard.edu
*This work was supported in part by the World Workshop in Oral Medicine V.
Received 22 December 2010; accepted 23 Deccember 2010

Keywords: oral; premalignancy; genetics

Introduction

With an annual incidence worldwide of over 500 000 cases, squamous cell carcinoma (SCC) of the head and neck region is the sixth most common malignancy today. Etiology of this malignancy within this anatomic region can be multi-factorial and site-specific. For example, oral cavity SCC (OSCC) is typically caused by chronic exposure to tobacco and alcohol (Blot *et al*, 1988). Historically, the etiology of oropharyngeal SCC has been similar to that of OSCC, namely tobacco use, with increased cancer incidence when alcohol abuse has also occurred. However, the paradigm of oropharyngeal SCC has fundamentally changed in recent years such that infection with high-risk subtypes of the human papillomavirus (HPV), particularly HPV-16, has emerged as a major causative factor for the neoplasm (Gillison *et al*, 2000, 2008; Schlecht, 2005; D'Souza *et al*, 2007; Chaturvedi *et al*, 2008; Fakhry *et al*, 2008). Thus, there are now fundamentally different molecular models of causation for OSCC as compared with oropharyngeal SCC.

There have also been impressive advances in recent years regarding the detection, prevention, and treatment of OSCC. Unfortunately, however, the overall 5-year survival for OSCC continues to be modest at best. OSCC survival is highly dependent on the stage of the tumor at diagnosis. For example, Stage I cancers have an ~80% 5-year survival rate while the survival rate decreases to ~20% for Stage IV lesions (Ries *et al*, 2008). To improve long-term outcomes therefore early detection in conjunction with primary and secondary prevention strategies is critical.

Screening and early detection are believed to decrease both the morbidity and mortality associated with OSCC because unlike many anatomic sites, oral cavity premalignant lesions are often visible upon clinical examination. However, accurate discrimination of premalignant *vs* reactive/inflammatory lesions via conventional visual and tactile examination alone is problematic. Notably, clinical presentation of oral premalignant lesions can be highly heterogeneous and may mimic more commonly occurring reactive/inflammatory mucosal lesions. As the malignant potential of oral lesions cannot be accurately predicted solely on the basis of their clinical characteristics, histologic evaluation is essential for all suspicious lesions. Biopsy results may reveal a range of tissue change, from a non-cancerous tissue condition to a precancerous lesion or frank malignancy.

As with the diagnosis of OSCC, a definition of oral mucosal premalignancy that is based upon conventional histologic examination can also be problematic. Lesions are currently considered precancerous when there are cytomorphologic changes consistent with dysplasia. However, the various criteria for diagnosing and grading dysplasia are controversial, highly subjective and open to a wide range of interpretation, even among highly qualified pathologists (Abbey *et al*, 1995; Warnakulasuriya *et al*, 2008). In addition, no definitive criteria currently exist for predicting risk of cancerous transformation of individual dysplastic lesions; even dysplastic oral lesions have been reported to undergo spontaneous regression.

Therefore, conventional histologic findings can only be utilized to indicate that a given lesion may have malignant potential, and cannot be used for the prediction of malignant change. Figure 1 underscores this concept and highlights two provocative yet key issues that are not prominently discussed in the literature:

- In general, OSCC progression may not occur in a linear fashion over a uniform period of time. Rather, there are subsets of lesions with histologic evidence of dysplasia that may or may not progress to OSCC.
- Similarly, histologically 'normal' appearing mucosal lesions may truly be benign or they may represent molecular premalignant lesions that have not yet developed morphologic/cytologic changes consistent with dysplasia.

Several recent studies underscore this concept. For example, a retrospective hospital-based study of 207 patients with dysplasia determined that during a 1-year follow-up, 39% of the lesions regressed, 20% remained stable, 33% developed new dysplastic lesions, and 7% developed OSCC (Arduino *et al*, 2009). Similarly, in a meta-analysis of 992 patients derived from 14 studies, Mehanna *et al* reported a malignant transformation rate of 12%, with a time to malignant transformation (TMT) of 4.3 years (Mehanna *et al*, 2009). Importantly, subgroup analyses by dysplastic grade and treatment modality showed no significant differences in TMT. Finally, Holmstrup *et al* reported that the estimated odds ratio for patients with oral dysplasia showed that none of the associated variables, including presence of any grade of dysplasia, had any influence on the risk of OSCC development (Holmstrup *et al*, 2006). These findings emphasize that, at the present time, it is not feasible to prognosticate accurately on the basis of histologic change. The data further highlight that development of molecularly based approaches to identify predictive biomarkers that could be used to interrogate lesions prior to and after the development of cytologic atypia would greatly improve the potential for early detection, prognostication and intervention.

Current modeling postulates that the development of cancer is driven by the accumulation of genetic and epigenetic changes within a clonal population of cells. These genotypic alterations can affect hundreds of genes, leading to phenotypic changes in critical cellular functions such as resistance to cell death, increased proliferation, induction of angiogenesis, and the ability to invade and metastasize. The mechanisms underlying these genetic and epigenetic aberrations include, but are not limited to, genomic instability through chromosomal rearrangement, amplification, deletion, methylation, and mutation. These genetic alterations have been shown to contribute directly to cancer development and progression and are central to understanding the biology of oncogenes and tumor suppressor genes (TSG) as well as the phenotypes they regulate. There has been considerable investigation into the genotypic and phenotypic alterations observed in OSCC. However, data regarding the incidence and timing of these changes in oral premalignancy are limited. Furthermore, it is not clear whether any of the genotypic changes, utilized individually or in a panel, can consistently predict which

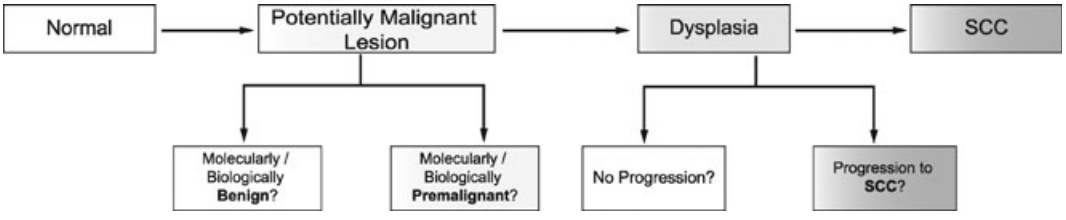

Figure 1 Oral lesion heterogeneity. Oral mucosal lesions, whether histologically normal or atypical, do not follow a linear progression pattern. Rather, a small majority of dysplastic lesions will progress to cancer, while the majority will either remain quiescent or even regress. Similarly, lesions lacking histologic atypia may represent reactive and molecularly benign lesions or they may be molecularly premalignant

dysplastic lesions will progress to OSCC. Therefore, the objectives of the review were:

1. To summarize the key genotypic and phenotypic alternations observed in potentially malignant oral lesions that are likely to progress to OSCC.
2. To determine whether these changes correlate with the clinicopathologic findings, and whether they aid clinical practice at this time.
3. To delineate future research directions that could enhance understanding of the pathobiologic model such that clinical management is strategically improved.

Search strategy
An initial search was done in Pubmed/Medline (1950 to January 2010) and Embase (1988 to January 2010) with overall search terms (keywords, text, and corresponding MeSH terms) that covered the genetics/epigenetics of oral dysplasia. This search was limited to meta-analyses, guidelines, reviews, and studies (without restrictions). *Addendum*. The initial search yielded a total of 16 meta-analyses, 4 guidelines, 145 reviews, and 1020 studies. All abstracts were reviewed by the authors and the search was refined with the key alterations as text terms 'aneuploidy', 'microRNAs (miRNA)', 'loss of heterozygosity', 'microsatellites', 'hypermethylation', 'telomerase', 'oncogenes', 'p53', 'tyrosine kinase', 'proliferation markers' cross referenced with 'oral dysplasia', 'premalignant oral lesions'. The purpose of this second phase was to narrow the focus to significant genetic/epigenetic events and progression of oral dysplasia. Manuscripts on viral factors, salivary gland disorders, lichenoid reactions, and lichen planus were excluded. References of selected review papers were analyzed for missed studies, and consultants to the review group (experts in the field) were queried for suggestions of significant literature to be included. A piloted data extraction form was used to abstract information of interest and selected papers were reviewed for strength of the evidence by assessing methodology, bias, and confounding factors.

Aneuploidy

Chromosomal instability often leads to imbalanced DNA content and the generation of near-diploid or aneuploid clones. Aneuploidy may result from gene dose imbalance, loss of TSG, gain of tumor promoting genes or oncogenes, or formation of fusion genes that leads to increased survival and proliferation advantage. Approximately 50–60% of oral cancers are aneuploid with one study reporting a figure of 90% (Diwakar *et al*, 2005; Abou-Elhamd and Habib, 2007; Torres-Rendon *et al*, 2009b). Aneuploidy in OSCC has also been shown to be associated with higher incidence of local recurrence and lymph node metastases (Baretton *et al*, 1995; Rubio Bueno *et al*, 1998; Hemmer *et al*, 1999). A study by Diwakar *et al* noted that while 52% of OSCC were aneuploid, 19% were heterogenous on ploidy studies (Diwakar *et al*, 2005).

Several studies have investigated the relationship between dysplasia and aneuploidy. Approximately 20–45% of oral dysplasia are aneuploid, with one study reporting a frequency of 83% (Pentenero *et al*, 2009; Torres-Rendon *et al*, 2009b; Donadini *et al*, 2010). Aneuploidy was found to be significantly and positively associated with the histologic grade of dysplasias (Saito *et al*, 1995; Pentenero *et al*, 2009; Donadini *et al*, 2010). Even mild dysplasias may show aneuploidy particularly if these are located on the tongue or floor of mouth (Islam *et al*, 2010). One study showed that aneuploid dysplasias were more likely to progress than diploid lesions (33% *vs* 11%, $P = 0.01$) and that aneuploid lesions transformed to OSCC within 5 years in 53% of cases *vs* 25% of diploid cases (Torres-Rendon *et al*, 2009b). Conversely, another study did not find a correlation between dysplasia and aneuploidy and the development of OSCC (Seoane *et al*, 1998). Two studies reported that proliferative verrucous leukoplakias may initially start as diploid lesions and develop aneuploidy over time prior to becoming OSCC (Kahn *et al*, 1994; Klanrit *et al*, 2007).

Summary and clinical significance: Aneuploidy is observed in 20–45% of oral dysplasias and there is some correlation between aneuploidy and histologic grade. In addition, there are limited and conflicting data regarding aneuploid dysplasias and the likelihood of progressing to OSCC. There are thus at the present time insufficient data to determine whether aneuploidy can be used as a biomarker for predicting the development of OSCC.

miRNA

The discovery of miRNA, 20–22 nucleotide-long members of the non-coding RNA family, adds another layer of gene regulation that is altered as cancer develops. They may be present as intergenic transcription units or found in the intronic sequences of protein-coding genes. More than 1,000 of these sequences have been identified and functional studies have identified that miRNAs act as conventional tumor suppressors or as oncogenes, and affect the translation or stability of target mRNA. Most are negative regulators of gene expression and have fundamental roles in biologic processes with this function being dysregulated as cancer develops. miRNAs of the let-7 family are examples of tumor suppressors with the genes mapping to chromosomal regions including the let-7g cluster at 3p21 (commonly deleted in squamous carcinomas). Validated target genes for miRNAs that function as TSG include Bcl-2, ras, myc, HMGA2, cyclin-dependent kinase 4 (CDK4), and CDK6, and target genes for miRNAs with ongogenic activity include PTEN, p27, p57, TIMP3, and BIM. miRNAs have been implicated in early disease. Cervigne *et al* found that miR-21, miR-181b, and miR-345 were consistently increased in oral dysplasia and associated with lesion severity (Cervigne *et al*, 2009). These regulatory sequences may have therapeutic potential as many of them influence multiple pathways that are dysregulated in cancer.

Summary and clinical significance: Presently, there is limited information regarding the expression of miRNAs in oral dysplasia. Insufficient evidence is available to delineate recommendations regarding the clinical utility of miRNA expression and the prediction of whether a dysplastic lesion will progress to OSCC.

Loss of heterozygosity (LOH) and microsatellite instability or allelic imbalance (AI)

Loss of heterozygosity and AI have been relevant targets in cancer research. AI may occur when one copy of a polymorphic marker (with two slightly different alleles) is lost (LOH) or amplified (allelic gain). The term LOH is commonly used to describe this process, but as allelic gain occurs very frequently, and may be more common, AI describes the process more accurately. AI occurs at loci across the genome at low frequency and at higher frequency at 3p (3p24–25, 3p21, 3p13–14), 9p21 (p16), 17p13 (p53) and 8p22–24, with loci at 13q14, 18q and 21q being implicated in some studies. The markers utilized in these studies evolved as more informative markers, and those showing higher frequency of AI (indicating the position of relevant genes), were discovered (el-Naggar *et al*, 1995; Califano *et al*, 1996, 2000; Mao *et al*, 1996; Roz *et al*, 1996; Partridge *et al*, 1997; Rosin *et al*, 2000; Tabor *et al*, 2001, 2003; Zhang *et al*, 2001a,b; Epstein *et al*, 2003; Garnis *et al*, 2004; Cheng and Wright, 2005). Consensus has emerged that AI at 3p and 9p provides useful evidence of the accumulation of genetic damage in potentially malignant lesions. This statement is based on studies performed at different laboratories and at distinct geographic locations where potentially malignant lesions are associated with different risk factors (el-Naggar *et al*, 1995; Califano *et al*, 1996, 2000; Mao *et al*, 1996; Partridge *et al*, 1997, 1998, 2000, 2001; Rosin *et al*, 1997, 2000, 2002; El-Naggar *et al*, 1998; Lee *et al*, 2000; Guo *et al*, 2001; Jiang *et al*, 2001; Kayahara *et al*, 2001; Poh *et al*, 2001; Tabor *et al*, 2001; Zhang *et al*, 2001a,b, 2005; Ha *et al*, 2002; Epstein *et al*, 2003; Garnis *et al*, 2004, 2005, 2009; Bremmer *et al*, 2005, 2008; Cheng and Wright, 2005; Tsui *et al*, 2008). At present, the identity of the relevant sequences at many of these loci is not known such that regulatory sequences as well as oncogenes or TSG may reside here. The role of mutant p53, as opposed to LOH, may be biologically relevant in view of the oncogenic activity of some mutant p53 proteins.

With respect to AI and dysplasia, initial studies (prior to 2002) revealed AI at many loci in different chromosomes (Califano *et al*, 1996; Partridge *et al*, 1997, 1998, 2000, 2001; El-Naggar *et al*, 1998; Rosin *et al*, 2000; Jiang *et al*, 2001; Tabor *et al*, 2001). In general, there is a trend for lesions with more disturbance in cellular organization and architecture to harbor more genetic changes at 3p and 9p (Califano *et al*, 1996, 2000; Mao *et al*, 1996; Roz *et al*, 1996; Tabor *et al*, 2001, 2003; Zhang *et al*, 2001a,b, 2005; Epstein *et al*, 2003; Tsui *et al*, 2008). However, not all studies confirm this observation and AI at 3p and 9p may not result in any phenotypic change in the oral epithelium that can be detected by light microscopy (Mao *et al*, 1996; Partridge *et al*, 2000, 2001; Jiang *et al*, 2001; Kayahara *et al*, 2001). It is clear, however, that progression may occur against a background of mild dysplasia especially when the lesion has a marked propensity for lateral spread. Cases presenting with widespread field change almost always harbor AI at 9p and frequently at 3p21, but the nature of the other events that result in clonal expansion and lateral spread is unclear (Califano *et al*, 1996; Partridge *et al*, 1997, 2001; Jiang *et al*, 2001; Tabor *et al*, 2001; Ha *et al*, 2002; Tsui *et al*, 2008).

Whether AI at 3p, 9p and 17p can predict risk of progression to dysplasia has not been conclusively established as only a few studies have compared the frequency of AI at the key loci for lesions in which carcinoma developed as well as for cases where progression did not occur. An increase in the frequency of AI at 3p and/or 9p for lesions with low-grade (mild and moderate) dysplasia was reported for 22 of 23 (96%) cases progressing to dysplasia, compared to 30/54 (56%) of non-progressing cases, with the risk of progression being greater when AI at any other of the chromosomes tested also occurred (Rosin *et al*, 2000). The relationship between increased AI and risk of progression was confirmed by case–control methodology using markers at 3p, 8p, 9p, and 13q, with progressing and non-progressing cases matched with respect to age, gender, site of the lesion, smoking, and alcohol habits together with the status of the margins (Partridge *et al*, 2000). In this report, AI LOH at 3p and 9p occurred in 35/39 (90%) of cases progressing and for 28/39 (72%) of those not progressing. Only LOH at 17p13 was significantly associated with risk of tumor development. These reports cannot be directly compared due to the different study designs and number of markers used. The study by Rosin *et al* (2000) used three markers at 3p and 19 at other chromosomes, whereas Partridge *et al* (2000) used 10 markers at 3p, 3 at 9p, and 9 at other chromosomes (Partridge *et al*, 2000; Rosin *et al*, 2000). Nevertheless, they confirm that information regarding increased frequency of AI provides indication that genetic damage is occurring and may be linked with risk of progression, but these markers cannot yet be used to predict risk of progression. Indeed, the link between AI at 3p and 9p and risk of progression, initially suggested by Mao *et al*, was weakened on long-term follow-up ($P = 0.09$), emphasizing the need for further prospective studies. In view of this, several studies have combined information derived from the application of multiple markers to improve the usefulness of a predictor (Califano *et al*, 2000; Lee *et al*, 2000; Partridge *et al*, 2001; Chen *et al*, 2005a). Additional chromosomes studied for LOH and AI are 8p, 8q, 11p, 17p, and 18q; however, the evidence supporting the predictive value of these loci is inconclusive (Califano *et al*, 1996, 2000; El-Naggar *et al*, 1998; Partridge *et al*, 1998, 2000; Jiang *et al*, 2001; Tabor *et al*, 2001, 2003; Rosin *et al*, 2002; Bremmer *et al*, 2005, 2008; Chen *et al*, 2005a).

Summary and clinical significance: There is preliminary albeit limited evidence in support of LOH and AI of

3p and 9p as early markers for dysplastic progression of premalignant oral lesions. Comparison among existent studies is challenged by methodologic differences, adjustment for confounders, and controls. The clinical utility of LOH in 3p and 9p as an effective screen for progression of oral premalignant lesion requires prospective validation.

Epigenetic events

Epigenetic changes involve modifications of DNA and histones that are not coded in the DNA sequence although these changes are heritable (Egger *et al*, 2004). Three systems are involved: DNA hypermethylation, RNA-associated post-transcriptional silencing, and histone modification. Of these, DNA methylation has been studied in OSCC. In normal tissues, unmethylated cytosine is found in high densities in CpG islands (areas with high concentration of cytosine and guanine) that map close to a promoter region in 40% of mammalian genes (Egger *et al*, 2004). This unmethylated state is associated with a high rate of transcriptional activity. Hypermethylation of TSG, mediated through the enzyme DNA methyltransferase, results in stable transcriptional silencing of tumor suppressor activity. This process has been detected in oral OSCC and is a hallmark of many other cancers as well.

In OSCC, hypermethylation of p16 occurs in 50–73% of cases and p15 in 60% of cases; (Wong *et al*, 2003; Goldenberg *et al*, 2004; Kulkarni and Saranath, 2004; Kato *et al*, 2006). Interestingly, 25–60% of 'normal margins' of resected specimens also showed a hypermethylated state (Wong *et al*, 2003; Kulkarni and Saranath, 2004; Kato *et al*, 2006). Only three studies investigated hypermethylation and oral dysplasia. Kresty *et al* identified hypermethylation of p16 INK4a and p14ARF in 57.5% and 3.8% of cases of severe dysplasia respectively (Kresty *et al*, 2002). Hypermethylation (as well as point mutation and deletion) of p16 (locus on 9p21) probably abrogates its activity via the p16/R6/cyclin D1 tumor suppressor pathway (Kresty *et al*, 2002; Goldenberg *et al*, 2004). Takeshima *et al* noted hypermethylation of p16 in 18% *vs* 55% of mild *vs* severe dysplasia; p14 in 77% mild *vs* 65% severe dysplasia; p15 in 50% milld *vs* 65% severe dysplasia and p53 in 32% mild *vs* 40% severe dysplasia (Takeshima *et al*, 2008). Patients with submucous fibrosis showed hypermethylation in 50–80% of these four markers. One study that investigated hypermethylation of RARB2 found 53% of oral leukoplakias were hypermethylated, but the histology of these lesions was not well defined (Youssef *et al*, 2004). Some of the other important genes shown to be hypermethylated in OSCC are the following (Youssef *et al*, 2004; Ha and Califano, 2006; Kato *et al*, 2006):

1. CDH1 (cadherin 1 Type 1, a gene on chromosome 16q22.1) that produces E-cadherin, which plays an important role in cell adhesion and contact inhibition.
2. MGMT gene (a gene on chromosome 10q26) which produces MGMT, O6-methylguanine-DNA-methyl transferase) a DNA repair enzyme that removes adducts caused by alkylating agents; such DNA repair causes the cells to be resistant to treatment-induced apoptosis. Silencing this gene thus allows alkylated guanine to accumulate leading to apoptosis (Kulkarni and Saranath, 2004; Kato *et al*, 2006).
3. DAPK1 (death-associated protein kinase-1), a TSP on chromosome 9q22 associated with apoptosis (Kulkarni and Saranath, 2004; Ha and Califano, 2006; Kato *et al*, 2006).
4. RARB2 gene (retinoic acid receptor B2 gene on chromosome 3p24) codes for the nuclear receptor that suppresses transcription activation and suppresses cell proliferation and squamous differentiation (Youssef *et al*, 2004).

Hypermethylation of p14ARK, p16 INK4a, P15, MGMT, DAPK, GSTP1 and RARB have been seen in dysplasias and in histologically normal appearing margins of OSCC resections. However, these studies do not correlate hypermethylated states with recurrence of OSCC or with progression to invasive OSCC. There is some evidence that hypermethylation of p14 is linked to p53 activity and that lesions on the tongue and floor of mouth, high-risk sites for dysplasia and malignancy, tend to be hypermethylated (Kresty *et al*, 2002). A few studies also showed hypermethylation of p15 and p16 in smokers and drinkers and in patients with leukoplakia, but these were not confirmed by histopathologic examination.

Summary and clinical significance: Hypermethylation is seen with relatively high frequency in OSCC as well as at tumor margins and in dysplasias. However, these studies do not show a correlation with the degree of dysplasia and do not predict the development of OSCC. At this time, there is insufficient evidence to determine if hypermethylation can be used as a predictive biomarker for the progression of dysplastic lesions.

Telomerase regulation

Telomeres are specialized areas of the distal end of chromosomes composed of chromatin formed by tandem repeats of the sequence TTAGGG bound to specific telomere-binding proteins. They are progressively shortened with each cell division, ultimately resulting in aging and senescence of cells. As telomere loss limits lifespan of cells, the loss also reduces the probability of cancer development. Telomerase is an enzyme that directs the synthesis and maintenance of these telomeres and is composed of hTR (human telomerase RNA, the RNA template), hTEP1 or TP1 (telomerase-associated protein 1) and hTERT (human telomerase reverse transcriptase) (Pannone *et al*, 2007). Cancer cells are able to stabilize telomeres by activating telomerase, thereby bypassing senescence and facilitating cell immortalization (Shay and Wright, 2010).

Depending on the assay utilized, telomerase activity is noted in 67–100% of OSCC (Sumida *et al*, 1998;

Pannone *et al*, 2007). Enhanced telomerase expression is seen in 50–100% of moderate and severe dysplasia (Miyoshi *et al*, 1999; Zhang and Zhang, 1999; Liao *et al*, 2000; Kim *et al*, 2001; Chen *et al*, 2007). Fujita and Yajima *et al* both showed that OSCC, OSCC margins, and dysplastic lesions have similar expression of telomerase activity (Fujita *et al*, 2004; Yajima *et al*, 2004). Liao *et al* and Miyoshi *et al* showed that telomerase activity increases from 0–50% in mild to 50–100% in moderate-to-severe dysplasia, and was highly expressed in OSCC, suggesting that the acquisition of activity was part of multi-step carcinogenesis (Miyoshi *et al*, 1999; Liao *et al*, 2000). However, the number of cases evaluated was small. Zhang *et al* showed similar findings with carcinoma in situ and OSCC showing the highest degree of telomerase activity, and suggesting that this was a late event during progression (Zhang and Zhang, 1999). The study by Chen *et al* was the only one that did not find differences in expression between mild, moderate and severe dysplasia and OSCC (Chen *et al*, 2007). Mutirangura *et al* demonstrated that non-dysplastic leukoplakias that progressed to OSCC were also associated with increased telomerase activity (Mutirangura *et al*, 1996).

Summary and clinical significance: Compared with normal mucosa, telomerase activity is increased in dysplasias and OSCC. The activation of telomerase appears to be a late change during progression, but the frequency of increased telomerase activity varies greatly from study to study. There are no studies that have attempted to correlate telomerase activity and progression with OSCC. At this time, there is insufficient evidence to determine if increased telomerase activity can be used as a predictive biomarker for dysplastic lesions.

Proliferation markers

It is generally accepted that increased cell proliferation is associated with the progression in the multistep process of carcinogenesis. Immunohistochemical methods of detecting proliferation markers, such as proliferating cell nuclear antigen (PCNA), minichromosome-maintenance protein 2 (MCM2) and Ki-67, have been widely used as possible indicators of genetic abnormalities typical of malignant progression. Ki67 antigen is one of the best known proliferation markers as its expression is seen in proliferating cells (G1, S, G2 phase), but not in resting cells (G0 phase). MCM2 is expressed throughout the cell cycle, including cells leaving G0 to enter into the early G1 phase, distinguishing then from Ki67. PCNA, another marker frequently used as a measure of the proliferation, is an essential factor both for replication and for repair of DNA. Dysregulation of Ki67, PCNA, and MCM2 protein expression has been observed in OSCC (Iamaroon *et al*, 2004; Fourati *et al*, 2009; Torres-Rendon *et al*, 2009a; Watanabe *et al*, 2010). There is conflicting evidence regarding their possible role as prognostic markers for OSCC (Xie *et al*, 1999; Kodani *et al*, 2003; Szelachowska *et al*, 2006). Increased suprabasilar Ki67 immunostaining may correlate with increasing grades of dysplasia (Kushner *et al*, 1997; Jordan *et al*, 1998; Schoelch *et al*, 1999b; Gonzalez-Moles *et al*, 2000; Loro *et al*, 2002; Tabor *et al*, 2003; Bortoluzzi *et al*, 2004; Takeda *et al*, 2006; Vered *et al*, 2009). Only one paper included in this review failed to show any correlation between expression levels of Ki67 and degree of dysplasia (Soria *et al*, 2001). Among the cited papers, only three incorporated hyperplastic tissues derived from inflammatory lesions. Importantly, in each of these studies, the proliferation indices of the reactive lesions were very similar to the dysplastic specimens (Loro *et al*, 2002; Takeda *et al*, 2006; Vered *et al*, 2009).

Only one paper included in this review described PCNA expression in oral dysplastic lesions.

The PCNA labeling index (LI) increased steadily with lesion progression from normal to acquistition of malignant features, with a statistically significant increase in the transition from moderate to severe dysplasia (Shintani *et al*, 2002). In an immunohistochemical analysis of MCM2, Kodani described a trend of increased LI with increasing degree of dysplasia. Furthermore, a significantly higher MCM2 LI was associated with the subgroup of patients developing OSCC from dysplasias (Kodani *et al*, 2001). Li *et al*, through a quantitative real time PCR, found MCM2 mRNA expression levels increased with increasing grades of dysplasia, with a highly significant difference between mild and moderate ($P < 0{,}001$), and between moderate and severe dysplasia ($P < 0.001$) (Li *et al*, 2008).

Summary and clinical significance: There is a good overall correlation between Ki67 expression and the degree of dysplasia. However, Ki67 expression would appear to have low specificity for predicting which dysplastic lesions are more likely to progress to cancer given that other oral mucosal conditions, both benign and inflammatory, can result in increased Ki67 staining. Data regrading the expression of PCNA and oral dysplastic lesions is limited. Therefore, no conclusion can be deduced for its potential use in clinical practice, and further studies are necessary. The limited data to date suggest that increased expression of MCM2 mRNA and protein correlates with increasing grades of dysplasia. Therefore, the potential usefulness of MCM2 as a prognostic marker of potentially malignant oral lesions requires further evaluation.

p53 family: p53, p63, p73, p21, p27

p53 is a TSG located on chromosome 17p13. p53 plays a major role in cell-cycle progression, cellular differentiation, DNA repair and apoptosis, and is regarded as a guardian of the genome. Loss of p53 function diminishes the regulation of cell cycle arrest and apoptosis, thereby altering the ability of cells to respond to stress or damage (such as DNA damage, hypoxia, and oncogene activation). This can subsequently lead to genomic instability and the accumulation of additional genetic alterations. p53 is the most commonly inactivated TSG in human cancer including OSCC (Vousden and Lane,

2007). Various genetic events can lead to inactivation of p53 including mutation, inactivation through interaction with a viral protein of 'oncogenic' HPV subtype, (such as HPV16 or HPV18), or through loss of one allele as a result of LOH (Gonzalez *et al*, 1995; Olshan *et al*, 1997; Nagpal *et al*, 2002). In normal cells, p53 protein levels are low due to the wild-type protein's short half-life and are essentially undetectable by immunohistochemistry (IHC) (Smeenk and Lohrum, 2010). Stabilizing mutations may cause an increased half-life for the protein, which frequently results in increased expression of mutant p53 in neoplastic cells. Association of p53 with other proteins that protect against degradation has also been shown to be responsible for the over-expression of p53. IHC expression of a mutant p53 protein has been correlated with increased risk for secondary tumors, early recurrence, metastatic spread, and resistance to chemotherapy or radiation therapy (Shin *et al*, 1996; Temam *et al*, 2000; Warnakulasuriya *et al*, 2000). Poeta *et al*, 2007 reported that inactivation of p53 in OSCC is associated with reduced survival after surgical treatment (Poeta *et al*, 2007). However, in view of the heterogeneity of laboratory techniques as well as limited clinical data of various studies, the value of the p53 as a biomarker in patients with OSCC is still controversial. In addition, the wide spectrum of p53 mutations observed among tumor samples suggests that the mutations vary in their prognostic power. It has been established by several studies that there is a role for p53 as a biomarker in dysplastic lesions; however, there is no consensus yet on the details. Various studies have reported the analysis of p53 expression by IHC based on the percentage of positive cells, the distribution of the cells within the epithelial lining and the intensity of the stain. However, correlation between expression of p53 and histologic grade of dysplasia has yielded inconsistent results. Bortoluzzi and Vered found that suprabasal p53 staining correlated with increasing histologic grade, whereas Schoelch reported that higher degree of dysplasia correlated with an increased percentage of p53 positive cells (Schoelch *et al*, 1999a; Bortoluzzi *et al*, 2004; Vered *et al*, 2009). Brennan and Kodani found that, in addition to percentage of positive cells, intensity of staining correlated with higher grades of dysplasia (Brennan *et al*, 2000; Kodani *et al*, 2001).

Cruz did not find a correlation between p53 expression and the degree of dysplasia (Cruz *et al*, 1998). However, they did find that suprabasal expression of p53 was an early event of malignant transformation and had predictive value for developing oral SCC ($P = 0.002$). Conversely, Murti *et al* found that p53 expression was not predictive of malignant transformation (Murti *et al*, 1998). In biopsies from cohorts of patients who did or did not progress to cancer over a period of up to 25 years, similar levels of p53 expression were found (29% and 31% respectively; $P > 0.05$). Kodani reported that increased p53 labeling indices (mean percentage of positive cells) may correlate with increased risk of malignant transformation, while Shah *et al*, found that overexpression of p53 represented the greatest risk for this outcome ($P = 0.0001$) (Kodani *et al*, 2001; Shah *et al*, 2007). Smith *et al*, who reviewed the literature regarding dysplasia, concluded that the pooled relative risk for cancer progression in p53 positive cases was 0.96, 0.65, 1.42) while for p53 staining in suprabasal layers, only the relative risk was higher (1.36, 0.27, 6.82). The *P* values for heterogeneity were 0.38 and 0.94 respectively (Smith *et al*, 2009).

p63 and p73 are members of the p53 family, and are related both structurally and functionally to p53 (Smeenk and Lohrum, 2010). Both cooperate with p53 to induce apoptosis, suggesting that they have a role in the regulation of DNA damage-induced cell death. The p63 gene, located at 3q27–29, is responsible for the transcription of six isoforms. Three isoforms contain an N-terminal transactivation (TA) domain and can induce apoptosis; the remaining three isoforms lack the TA domain, and may function in a dominant-negative fashion as oncoproteins. There are limited data regarding the p63 and p73 expression and OSCC (Bortoluzzi *et al*, 2004). p63 gene amplification has been associated with prognostic outcome in OSCC (Thurfjell *et al*, 2005). Conversely, Oliveira *et al* found that p63 was over expressed in the majority of OSCC (64.4%), but with conflicting results regarding its association with survival (Oliveira *et al*, 2008). In normal and hyperplastic mucosa, p63 protein expression was limited to the basal and parabasal layers, while its expression extended to the more superficial layers with increasing grades of dysplasia (Chen *et al*, 2005b; Takeda *et al*, 2006; Vered *et al*, 2009). Conversely, Bortoluzzi found neither typical tendency nor statistically significant differences in the p63 staining score among the 3 grades of dysplasia (Bortoluzzi *et al*, 2004). p73 is a member of the p53 family and located on chromosome 1p36 that frequently demonstrates deletions and is thought to contain multiple TSG. In one study, p73 protein expression was detected in basal cells of normal epithelium and showed a significant suprabasal expression in dysplasias. However, there was no difference in p73 expression with increasing histologic grade of dysplasia (Chen *et al*, 2004).

The CDK inhibitor (CDKI) p21 mediates growth arrest following DNA damage by inactivating members of the cyclin family. Altered expression of p21 in OSCC has been reported with respect to its correlation with tumor biology and clinical outcomes (Erber *et al*, 1997; Venkatesan *et al*, 1999; Xie *et al*, 2002; Nemes *et al*, 2005). Choi *et al* reported that p21 expression increased during histologic progression, but that there no was significant correlation between expression and progression to OSCC (Choi *et al*, 2003). Similarly, Shintani reported a correlation between the degree of dysplasia and histologic progression, and positive staining in 3% of mild, 50% of moderate, and 64% of severe dysplastic samples (Shintani *et al*, 2002). Similarly, Schoelch *et al* reported an increasing trend in levels of p21 expression from normal to dysplastic to malignant mucosa (Schoelch *et al*, 1999b). Conversely, Kodani reported a decreasing trend in p21 expression with increasing histologic grade. Specifically, the authors reported that the progression to OSCC from dysplasia correlated with

significantly lower levels of p21 expression (Kodani *et al*, 2001). There are no published data that correlate p21 expression and prediction of progression with OSCC.

The p27 gene located on chromosome shares sequence homology with p21, and acts as a p53 independent negative cell cycle regulator involved in G1 arrest. It associates with several CDK complexes, resulting in loss of their activity and inability to phosphorylate the retinoblastoma (Rb) gene. Although rarely mutated, reduced levels of p27 protein expression have been reported in various human tumors including OSCC (Lee and Kim, 2009). In general, data for OSCC suggest that there is decreased p27 expression when compared with normal tissue and that this decrease is associated with poor prognosis (Jordan *et al*, 1998; Venkatesan *et al*, 1999; Kudo *et al*, 2000a; Kuo *et al*, 2002; Shintani *et al*, 2003). However, several manuscripts have reported increased p27 expression in OSCC (Fujieda *et al*, 1999; Kudo *et al*, 2000b). With respect to oral premalignancy, several papers have described a positive suprabasal staining pattern in normal and mild dysplasia that became less apparent with increasing degrees of dysplasia (Jordan *et al*, 1998; Schoelch *et al*, 1999b; Kodani *et al*, 2001; Shintani *et al*, 2002). However, there are insufficient data to determine if altered p27 expression can be used to predict malignant transformation.

Summary and clinical significance: Expression of p53 is observed in dysplastic lesions and its expression is correlated with increasing histologic grade. There are conflicting data regarding the value of p53 as a predictor for progression to OSCC. There are insufficient data to determine if p63 or p73 expression can be used to identify which dysplastic lesions are more likely to progress to OSCC. To evaluate fully the diagnostic utility of the p53 family, future studies must consider the pathway as a whole as well as each of the various mechanisms by which a particular gene/protein in the pathway may be inactivated. There are conflicting reports regarding the expression of p21, although majority of data suggest that its expression is lost during progression to cancer. There is currently insufficient information to determine if p21 can predict which dysplastic lesions will progress to cancer. Expression of p27 appears to decrease with increasing grades of dysplasia. However, its utility as a diagnostic biomarker is unknown.

Rb family: Rb, p16

The Rb gene was the first TSG identified and plays a key role in the regulation of cellular proliferation. There is compelling evidence that several components of the Rb pathway are altered in human cancer, including OSCC (Todd *et al*, 2002). The expression of Rb protein in OSCC has been variably reported. Many studies have reported loss of Rb protein expression, while others have showed elevated levels of expression (Yoo *et al*, 1994; Pande *et al*, 1998; Xu *et al*, 1998; Schoelch *et al*, 1999b; Nakahara *et al*, 2000). There are limited data regarding the expression of Rb in premalignant lesions. Soni *et al* reported a significant loss of pRb at the transition from hyperplasia to dysplasia (Soni *et al*, 2005).

Inactivation of the p16 TSG is a common event in various types of cancer and may be one of the first TSG inactivated in OSCC. Inactivation of the gene may have prognostic relevance (Reed *et al*, 1996; Papadimitrakopoulou *et al*, 1997; Pande *et al*, 1998; Xu *et al*, 1998; Lai and El-Naggar, 1999; Nakahara *et al*, 2000; Muirhead *et al*, 2006; Suzuki *et al*, 2006). P16 is a member of a family of negative cell cycle regulatory proteins that inhibits the activity of cyclin D–CDK4/6 complexes, thereby preventing Rb phosphorylation. Data regarding the direction (overexpression or loss) of immunohistochemical detection of p16 expression in OSCC are conflicting. Gologan *et al* found a significant correlation between an increase in expression of p16 and increasing degrees of dysplasia (Gologan *et al*, 2005). Similarly, Chen observed an increasing trend of p16 staining score with increasing grades of dysplasia (Chen *et al*, 1999). Conversely, several investigators have found a significant correlation between decreased p16 protein expression and histologic grade of dysplasia (Soria *et al*, 2001; Shintani *et al*, 2002; Bradley *et al*, 2006). While not the focus of this review, it should be noted that overexpression of p16 in SCC is now used as a surrogate for identifying HPV-associated SCC *vs* SCC associated with tobacco and alcohol consumption. This is particularly relevant in non-oral sites such as the tonsil, base of tongue and oropharynx.

Summary and clinical significance: There are limited reports regarding the expression of Rb in dysplasia. Furthermore, there are insufficient data regarding the ability of Rb protein expression to predict progression to OSCC. There are conflicting data regarding the expression of p16 in dysplastic lesions. The data are also insufficient to determine whether p16 expression (either gain or loss) is predictive of progression to OSCC. Further research is thus required to define more comprehensively the diagnostic/predictive potential of the Rb pathway.

Receptor tyrosine kinase pathways

Several signal transduction pathways are frequently altered in cancer and share common nodes and interact as a network. Their modification can affect cell survival, cell proliferation, morphology, and angiogenesis. Comprehension of the underlying pathways governing the progression of oral premalignant lesions is thus of utmost importance. A number of growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), and transforming growth factor-α (TGF-α) family members, signal by inducing dimerization and activation of receptors that are protein tyrosine kinases. Regulation of normal epithelium by growth factors such as TGF-α or EGF

is dependent on the expression of the corresponding receptors on the target cell.

Receptor tyrosine kinase pathway (EGFR/TGF-α)
The EGF receptor (EGFR) pathway plays an important role in cell proliferation, apoptosis, invasion, angiogenesis, and metastasis. Via the tyrosine kinase cascade, the receptor tyrosine kinase (also known as Type I receptor tyrosine kinases or ErbB tyrosine kinase receptors) has many downstream signaling targets that are associated with carcinogenesis. Development, growth, and survival of OSCC are highly dependent upon the EGFR signaling pathway. Increased expression of EGFR and TGF-α is observed in most OSCC, and expression correlates with poor prognosis (Ciardiello and Tortora, 2003). EGFR signaling also appears to be important at the stage of oral premalignancy. For example, Grandis *et al* found increased expression of TGF-α and EGFR mRNA expression in both dysplasias and OSCC (Grandis and Tweardy, 1993). Furthermore, amplification of EGFR in premalignancy has also been described. Specifically, Nagatsuka *et al* reported EGFR amplification in epithelial dysplasia and carcinoma in situ, and this amplification increased with the histologic grade of dysplasia (Nagatsuka *et al*, 2001). Similarly, using IHC, several investigators have reported increased expression of both EGFR and TGF-α in premalignant lesions (Bergler *et al*, 1989; Shin *et al*, 1994; Christensen, 1998; Srinivasan and Jewell, 2001). These findings suggest an increased receptor–ligand interaction with increasing degrees of oral dysplasia. Therefore, coexpression of TGF-α and EGFR may provide an early marker for the onset of epithelial dysplasia preceding OSCC. These results also suggest that EGFR expression may be suitable as a potential intermediate endpoint in evaluating the efficacy of oriented target therapies trials. In a recent study, Taoudi Benchekroun *et al* assessed whether EGFR expression and gene copy number changes might predict the risk of progression of oral leukoplakia to oral SCC. They reported that increased EGFR gene copy number in oral leukoplakias was associated with an increased risk of developing OSCC (Taoudi Benchekroun *et al*, 2010).

Summary and clinical significance: Increased EGFR and TGF-α are observed in both premalignancy and OSCC. Recent data from a single study have reported that EGFR copy number may correlate with malignant transformation. Further studies are required to validate the utility of EGFR as a predictive biomarker.

Phosphoinositide 3-kinase (PI3K)/AKT pathway
Once activated, EGFR stimulates a number of downstream signaling events, namely the Ras/Raf/mitogen activated protein kinase (MAPK) signaling pathway, the transcription factor signal transducer and activator transcription, and the PI3K/AKT pathway, which in turn contributes to the malignant growth, and metastatic potential of oral cancer (Molinolo *et al*, 2009). PI3K is a lipid kinase that phosphorylates structural components of the cell membrane such as the inositol of phosphatidyl-1D-myo-inositol (PI) at the 3-position, and is known to be closely involved in carcinogenesis (Massarelli *et al*, 2005). Activation of PI3K by growth factors generates PI(3,4)P2 and PI(3,4,5)P3, which act as second messengers. PI(3,4,5)P3 causes activation of PH domain-containing proteins AKT and PDK1—AKT upstream kinase—thus enabling PI3K to transmit signals related to cell survival and proliferation (Fujita and Tsuruo, 2003).

Watanabe *et al*, reported that mRNA expression of PI3K class III was 2.5–11 times greater in dysplastic mucosa and OSCC compared with normal tissue. This was further validated by IHC by demonstrating the presence of p-AKT-positive cells only in dysplastic and early cancerous lesions (Watanabe *et al*, 2009). This finding is substantiated by Kaur *et al*, who assessed PI synthase expression by IHC in clinical specimens from oral leukoplakias without dysplasia, with dysplasia (mild, moderate and severe) and OSCCs. They reported increased PI synthase expression to be an early event in oral tumorigenesis, further sustained during the development and progression of OSCC (Kaur *et al*, 2010). AKT activation has been shown as an early event in oral preneoplastic lesions, and its expression is correlated with poor outcome in oral cancer patients (Massarelli *et al*, 2005). Massarelli *et al*, reported frequent expression of p-AKT in oral dysplasia, with AKT activation being found in 55% of the cases which progressed to OSCC (Massarelli *et al*, 2005).

Summary and clinical significance: These data further support the assertion that the PI3K-AKT signal pathway is closely related to oral precancerous lesions as well as the development of OSCC (Amornphimoltham *et al*, 2004). Nevertheless, despite the evidence, large-scale studies are warranted to evaluate further the PI3-K/AKT pathway's potential as an indicator of progression risk in leukoplakia and a role in development and progression during early stages of oral tumorigenesis.

ERK/MAPK pathway
The extracellular-signal regulated kinases (ERK/MAP-Ks) pathway is critically involved in the regulation of cell differentiation, proliferation, and survival (Mishima *et al*, 2002). MAPKs are activated by phosphorylation on two sites within the kinase domain and activated forms phosphorylate serine/threonine residues present on effector kinases. The MAPKs include two mammalian isoforms (ERK1, p44MAPK and ERK2, 42MAPK), which are translocated to the nucleus upon activation by growth factors such as EGF, NGF, and PDGF (Marshall, 1995).

Extracellular-signal regulated kinases/mitogen activated protein kinases play a central role in mitogenic signaling, which is a cascade of phosphorylation reactions involving cell surface receptor, Ras, Raf, and MEK or protein kinase C, Raf, and MEK (Cobb and Goldsmith, 1995; Gutkind, 1998). Activation of ERK1/2-MAPK pathway is often the result of the stimulation of EGFR signaling, with previous studies showing that in OSCC the Ras/RAF/MAPK pathway may be either constitutively activated due to gain in functional mutations in ras genes or may be activated

downstream from the persistent autocrine or paracrine stimulation of EGFR and other growth factor receptors, namely FGF (Tsui *et al*, 2009). Tsui *et al* performed whole genome DNA microarray analysis of 50 dysplastic lesions (21 CIS, 22 severe dysplasias, six mild/moderate dysplasia and one hyperplasia) that later progressed to cancer. They reported that 40% of the dysplastic lesions and 43.5% of the OSCC exhibited DNA amplification and homozygous deletions (Tsui *et al*, 2009). Furthermore, they reported that 25 oral lesions (one progressing low-grade lesion, 14 high-grade dysplasias and 10 oral SCC) showed a high-level gene amplification of different genes inside a signaling network which included the canonical ERK/MAPK, FGF, p53, PTEN and PI3K/AKT signaling pathways. While 34% of the low-grade lesions that progressed to OSCC contained these alterations, no similar changes were found in the low-grade dysplasias that did not progress (Tsui *et al*, 2009).

Summary and clinical significance: There is evidence of ERK/MAPK alterations in dysplastic lesions of the oral cavity. Furthermore, there is some evidence that alterations in this pathway may help identify a subset of dysplastic lesions that are more likely to progress to OSCC. Additional studies are required to determine their diagnostic and prognostic utility.

Cyclin D1 pathway

The CCND1 gene encodes the cyclin D1 protein which is a key regulator of the G1 phase of the cell cycle. Deregulation of the cell cycle is linked to carcinogenesis, specifically, the deregulation of G1–>S phase progression. The transition from G1 into S phase is regulated by CDKs, CDK4 and CDK6, in protein complexes with cyclin D1 (Huang *et al*, 2006). Cyclin D1 catalyzes the phosphorylation of Rb, which then releases the transcriptional factor E2F that will activate a number of downstream genes necessary for cell cycle progression. Therefore, overexpression of the protein accelerates the G1 phase transition, whereas inhibition of cyclin D1 results in cell cycle arrest.

Overexpression of cyclin D1 is the result of gene rearrangement and gene amplification and is often present in OSCC. For example, Mineta *et al* reported that OSCC demonstrating overexpression of cyclin D1 had a 39% 5-year survival (Mineta *et al*, 2000). With respect to oral premalignancy, Kövesi *et al* reported that cyclin D1 expression increased in parallel to the severity of dysplasia (Kovesi and Szende, 2006). Turatti *et al* also reported that the major components AP-1 transcriptional factors (c-Jun and c-Fos) and cyclin D1 are altered in dysplastic epithelium and OSCC, with cyclin D1 expression increasing with the degree of histologic differentiation from normal to moderate dysplasia and OSCC (Turatti *et al*, 2005). Ishida *et al* assessed the relationship between the expression of components of the canonical Wnt pathway and the progression of dysplasia in oral leukoplakia and showed that two of the target genes of the Wnt/β-cat pathway—c-Myc and Cyclin D1—were overexpressed in leukoplakia. They reported an increase in cyclin D1 expression during histologic progression of the severity in oral leukoplakia, with overexpression being more evident in oral leukoplakia with dysplasia than that without dysplasia (Ishida *et al*, 2007). Ye *et al* used a pathway-based approach to assess the complex interaction between single-nucleotide polymorphisms (SNPs) from genes in the cell-cycle control pathway, smoking status, and the overall risk of oral premalignant lesions. They reported that the CCND1 P241P polymorphism was significantly associated with a 2.5-fold increased risk of oral premalignant lesion (Ye *et al*, 2008). Huang *et al* also assessed the role of cell-cycle deregulation in carcinogenesis. They genotyped CCND1 SNPs in a case–control study of 115 oral premalignant lesions and 230 controls and showed that individuals with one or more copies of the CCND1 G870A variant A-allele had an increased risk of oral premalignant lesion development. These findings support the hypothesis that this polymorphism may be a susceptibility factor for OSCC (Huang *et al*, 2006).

Summary and Clinical Relevance: Alterations in the Cyclin D1 pathway are present in both OSCC and dysplasia. However, there is currently insufficient evidence to determine whether these alterations could be used as predictive markers to identify which dysplasias are more likely to progress to OSCC.

Vascular endothelial growth factor (VEGF) pathway

Angiogenesis is an essential phenotype in both physiologic and pathologic settings including tumor formation. The angiogenic phenotype is one of the first recognizable phenotypic changes observed in both experimental models as well as in human OSCC, suggesting that angiogenesis markers may hold promise for diagnosis and prevention (Pazouki *et al*, 1997; Macluskey *et al*, 2000; Carlile *et al*, 2001). The VEGF family is thought to be one of the factors that play a central role in the induction of blood vessel growth. VEGF acts by increasing vessel permeability and enhancing endothelial cell proliferation, migration and differentiation (Tae *et al*, 2000). The biologic effects of the VEGF ligands are mediated through their binding to members of the VEGF receptor family (VEGFR-1, VEGFR-2, VEGFR-3).

Vascular endothelial growth factor expression is increased in both dysplasia and HNSCC (Inoue *et al*, 1997; Moriyama *et al*, 1997; Shintani *et al*, 2004; Li *et al*, 2005; Johnstone and Logan, 2007). With respect to premalignancy, Johnstone *et al* reported a significant up-regulation of VEGF during progression from normal oral mucosa to dysplasia and OSCC (Johnstone and Logan, 2006, 2007). However, they found no correlation between VEGF expression and the grade of dysplasia (Denhart *et al*, 1997; Johnstone and Logan, 2007). This was consistent with the results of Carlile *et al* who observed that the increase in the histologic grade of dysplasia was not necessarily accompanied by an increase in VEGF expression (Carlile *et al*, 2001). Conversely, Denhart *et al* reported that only 50% of premalignant lesions and 75% of OSCC expressed VEGF (Denhart *et al*, 1997; Johnstone and Logan, 2007), implying that 50% of the premalignant and 25%

of the malignant lesions in this study were inducing angiogenesis via an alternative mechanism(s) that did not seem to involve VEGF. Similarly, Tae *et al* found that levels of VEGF in premalignant and malignant oral tissue were lower than in normal tissue (Tae *et al*, 2000). Finally, Hasina *et al* reported that OSCC demonstrate angiogenic heterogeneity that had an impact on targeted anti-angiogenic therapy (Hasina *et al*, 2008).

Summary and clinical significance: The current data suggest that there is heterogeneity with respect to the expression of VEGF in both dysplasia and OSCC. These findings suggest that selection of a single angiogenic factor/pathway biomarker may have limited ability to predict which lesions may or may not progress to OSCC.

Future research directions

The overarching purpose of this article has been to review the current state of knowledge of the genetic/epigenetic alterations that are specifically observed in oral mucosal premalignancy. The ultimate goal of this research is to identify the specific candidate biomarkers that would have optimal predictive capacity for identification of those dysplastic lesions most likely to progress to OSCC over time.

Analysis of correlations between biomarkers, stages of dysplasia and their progression to OSCC is complex and requires incorporation of multiple variables. The literature published to date delineates two primary, interrelated approaches for study of this model:

1. A description of biomarker profiles at a specific point in time, including expression and pattern of distribution, in relation to grade of dysplasia.
2. Correlation of changes of biomarker profiles over time in relation to progression of dysplasia, and, in a subset of lesions, to cancer.

The potential to identify accurately and prospectively the subset of dysplastic lesions likely to progress through dysplasia to cancer is of premier scientific and clinical significance. Biomarker profiles are of high value in this regard and may ultimately supercede histopathologic staging in the future. Further research is needed to delineate this paradigm more fully.

Selected published studies are based on the assumption that the more advanced the degree of dysplasia, the higher the likelihood of progression to frank cancer. However, this assumption is not fully substantiated in the literature.

Reports of time from progression of dysplasia to malignancy vary considerably across studies and the, majority of dysplastic lesions do not undergo malignant transformation, even after many years. Nevertheless, it is likely that even a 1–5% rate of transformation from dysplasia to OSCC would be of concern to most patients. New research is needed that integrates histopathology, biomarkers and molecular profiling with well-established clinical parameters including comprehensive baseline data, homogeneous patient populations and long-term follow-up.

The current state of the science has thus provided the foundation to pursue several key research issues, including the following:

1. Standardization of the definition of dysplasia, including whether the dysplasia is occurring in the absence of invasive cancer or is contiguous with tumor margin. These two types of lesions may have similar clinical and histopathologic appearance, but etiopathogenesis of the processes may be fundamentally different.
2. Standardization of the definition of control tissues used in comparison with dysplastic or OSCC tissue. For example, control tissue could be collected from normal appearing oral mucosa in the patient with dysplasia at another oral site or from a healthy subject with no documented dysplasia or cancer. Benign lesions (e.g., frictional hyperkeratosis, fibroma) as well as benign inflammatory lesions should be considered in this modeling as well to insure that the expression of the biomarker in question is not also seen in reactive lesions.
3. Consideration of expression of each biomarker by either distribution pattern (e.g., basal or suprabasal sites) or indices (e.g., percentage) of positive staining cells. In addition, potential impact of variability in laboratory technology including antibody staining techniques should be addressed.
4. Key elements related to the clinical cohorts, including:
   - Inclusion and exclusion criteria;
   - Patient risk behaviors, including tobacco use, alcohol abuse, and HPV status;
   - Length of long-term follow-up.
5. Correlation of histologic findings with the clinical appearance of oral mucosal lesions. The translational research opportunities associated with these and related key research issues will be described in a companion paper from our group. This latter manuscript will incorporate additional detailed modeling associated with the design strategy in relation to the state-of-the-science evidence base.

Author contributions

Lingen MW, Pinto A, Mendes RA, Franchini R, Czerninski R, Tilakaratne WM, Partridge M, Peterson DE, and Woo S-B participated in the discussion of content, the review of articles and the preparation of the manuscript.

References

Abbey LM, Kaugars GE, Gunsolley JC *et al* (1995). Intraexaminer and interexaminer reliability in the diagnosis of oral epithelial dysplasia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **80:** 188–191.

Abou-Elhamd KE, Habib TN (2007). The flow cytometric analysis of premalignant and malignant lesions in head and neck squamous cell carcinoma. *Oral Oncol* **43:** 366–372.

Amornphimoltham P, Sriuranpong V, Patel V *et al* (2004). Persistent activation of the Akt pathway in head and neck squamous cell carcinoma: a potential target for UCN-01. *Clin Cancer Res* **10:** 4029–4037.

Arduino PG, Surace A, Carbone M *et al* (2009). Outcome of oral dysplasia: a retrospective hospital-based study of 207 patients with a long follow-up. *J Oral Pathol Med* **38:** 540–544.

Baretton G, Li X, Stoll C, Fischer-Brandies E, Schmidt M, Lohrs U (1995). Prognostic significance of DNA ploidy in oral squamous cell carcinomas. A retrospective flow and image cytometric study with comparison of DNA ploidy in excisional biopsy specimens and resection specimens, primary, tumors, and lymph node metastases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **79:** 68–76.

Bergler W, Bier H, Ganzer U (1989). The expression of epidermal growth factor receptors in the oral mucosa of patients with oral cancer. *Arch Otorhinolaryngol* **246:** 121–125.

Blot WJ, McLaughlin JK, Winn DM *et al* (1988). Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res* **48:** 3282–3287.

Bortoluzzi MC, Yurgel LS, Dekker NP, Jordan RC, Regezi JA (2004). Assessment of p63 expression in oral squamous cell carcinomas and dysplasias. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **98:** 698–704.

Bradley KT, Budnick SD, Logani S (2006). Immunohistochemical detection of p16INK4a in dysplastic lesions of the oral cavity. *Mod Pathol* **19:** 1310–1316.

Bremmer JF, Braakhuis BJ, Ruijter-Schippers HJ *et al* (2005). A noninvasive genetic screening test to detect oral preneoplastic lesions. *Lab Invest* **85:** 1481–1488.

Bremmer JF, Braakhuis BJ, Brink A *et al* (2008). Comparative evaluation of genetic assays to identify oral pre-cancerous fields. *J Oral Pathol Med* **37:** 599–606.

Brennan PA, Conroy B, Spedding AV (2000). Expression of inducible nitric oxide synthase and p53 in oral epithelial dysplasia. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **90:** 624–629.

Califano J, van der Riet P, Westra W *et al* (1996). Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res* **56:** 2488–2492.

Califano J, Westra WH, Meininger G, Corio R, Koch WM, Sidransky D (2000). Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. *Clin Cancer Res* **6:** 347–352.

Carlile J, Harada K, Baillie R *et al* (2001). Vascular endothelial growth factor (VEGF) expression in oral tissues: possible relevance to angiogenesis, tumour progression and field cancerisation. *J Oral Pathol Med* **30:** 449–457.

Cervigne NK, Reis PP, Machado J *et al* (2009). Identification of a microRNA signature associated with progression of leukoplakia to oral carcinoma. *Hum Mol Genet* **18:** 4818–4829.

Chaturvedi AK, Engels EA, Anderson WF, Gillison ML (2008). Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. *J Clin Oncol* **26:** 612–619.

Chen Q, Luo G, Li B, Samaranayake LP (1999). Expression of p16 and CDK4 in oral premalignant lesions and oral squamous cell carcinomas: a semi-quantitative immunohistochemical study. *J Oral Pathol Med* **28:** 158–164.

Chen YK, Hsue SS, Lin LM (2004). p73 expression for human buccal epithelial dysplasia and squamous cell carcinoma: does it correlate with nodal status of carcinoma and is there a relationship with malignant change of epithelial dysplasia? *Head Neck* **26:** 945–952.

Chen L, Wong MP, Cheung LK, Samaranayake LP, Baum L, Samman N (2005a). Frequent allelic loss of 21q11.1 approximately q21.1 region in advanced stage oral squamous cell carcinoma. *Cancer Genet Cytogenet* **159:** 37–43.

Chen YK, Hsue SS, Lin LM (2005b). Expression of p63 protein and mRNA in oral epithelial dysplasia. *J Oral Pathol Med* **34:** 232–239.

Chen HH, Yu CH, Wang JT *et al* (2007). Expression of human telomerase reverse transcriptase (hTERT) protein is significantly associated with the progression, recurrence and prognosis of oral squamous cell carcinoma in Taiwan. *Oral Oncol* **43:** 122–129.

Cheng YS, Wright JM (2005). Oral and maxillofacial pathology case of the month. Focal cemento-osseous dysplasia. *Tex Dent J* **122:** 986–987.

Choi HR, Tucker SA, Huang Z *et al* (2003). Differential expressions of cyclin-dependent kinase inhibitors (p27 and p21) and their relation to p53 and Ki-67 in oral squamous tumorigenesis. *Int J Oncol* **22:** 409–414.

Christensen ME (1998). The EGF receptor system in head and neck carcinomas and normal tissues. Immunohistochemical and quantitative studies. *Dan Med Bull* **45:** 121–134.

Ciardiello F, Tortora G (2003). Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. *Eur J Cancer* **39:** 1348–1354.

Cobb MH, Goldsmith EJ (1995). How MAP kinases are regulated. *J Biol Chem* **270:** 14843–14846.

Cruz IB, Snijders PJ, Meijer CJ *et al* (1998). p53 expression above the basal cell layer in oral mucosa is an early event of malignant transformation and has predictive value for developing oral squamous cell carcinoma. *J Pathol* **184:** 360–368.

Denhart BC, Guidi AJ, Tognazzi K, Dvorak HF, Brown LF (1997). Vascular permeability factor/vascular endothelial growth factor and its receptors in oral and laryngeal squamous cell carcinoma and dysplasia. *Lab Invest* **77:** 659–664.

Diwakar N, Sperandio M, Sherriff M, Brown A, Odell EW (2005). Heterogeneity, histological features and DNA ploidy in oral carcinoma by image-based analysis. *Oral Oncol* **41:** 416–422.

Donadini A, Maffei M, Cavallero A *et al* (2010). Oral cancer genesis and progression: DNA near-diploid aneuploidization and endoreduplication by high resolution flow cytometry. *Cell Oncol* **32:** 373–383.

D'Souza G, Kreimer AR, Viscidi R *et al* (2007). Case–control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med* **356:** 1944–1956.

Egger G, Liang G, Aparicio A, Jones PA (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429:** 457–463.

El-Naggar AK, Coombes MM, Batsakis JG, Hong WK, Goepfert H, Kagan J (1998). Localization of chromosome 8p regions involved in early tumorigenesis of oral and laryngeal squamous carcinoma. *Oncogene* **16:** 2983–2987.

Epstein JB, Zhang L, Poh C, Nakamura H, Berean K, Rosin M (2003). Increased allelic loss in toluidine blue-positive oral premalignant lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **95:** 45–50.

Erber R, Klein W, Andl T *et al* (1997). Aberrant p21(CIP1/-WAF1) protein accumulation in head-and-neck cancer. *Int J Cancer* **74:** 383–389.

Fakhry C, Westra WH, Li S *et al* (2008). Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *J Natl Cancer Inst* **100:** 261–269.

Fourati A, El May MV, Ben Abdallah M *et al* (2009). Prognostic evaluation of p53, heat shock protein 70, Ki67, and CD34 expression in cancer of the tongue in Tunisia. *J Otolaryngol Head Neck Surg* **38:** 191–196.

Fujieda S, Inuzuka M, Tanaka N *et al* (1999). Expression of p27 is associated with Bax expression and spontaneous apoptosis in oral and oropharyngeal carcinoma. *Int J Cancer* **84:** 315–320.

Fujita N, Tsuruo T (2003). Survival-signaling pathway as a promising target for cancer chemotherapy. *Cancer Chemother Pharmacol* **52**(Suppl 1)**:** S24–S28.

Fujita H, Nagata M, Hoshina H *et al* (2004). Clinical significance and usefulness of quantification of telomerase activity in oral malignant and nonmalignant lesions. *Int J Oral Maxillofac Surg* **33:** 693–699.

Garnis C, Coe BP, Ishkanian A, Zhang L, Rosin MP, Lam WL (2004). Novel regions of amplification on 8q distinct from the MYC locus and frequently altered in oral dysplasia and cancer. *Genes Chromosomes Cancer* **39:** 93–98.

Garnis C, Rosin MP, Zhang L, Lam WL (2005). Alteration of AKAP220, an upstream component of the Rb pathway, in oral carcinogenesis. *Int J Cancer* **116:** 813–819.

Garnis C, Chari R, Buys TP *et al* (2009). Genomic imbalances in precancerous tissues signal oral cancer risk. *Mol Cancer* **8:** 50.

Gillison ML, Koch WM, Capone RB *et al* (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* **92:** 709–720.

Gillison ML, D'Souza G, Westra W *et al* (2008). Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst* **100:** 407–420.

Goldenberg D, Harden S, Masayesva BG *et al* (2004). Intraoperative molecular margin analysis in head and neck cancer. *Arch Otolaryngol Head Neck Surg* **130:** 39–44.

Gologan O, Barnes EL, Hunt JL (2005). Potential diagnostic use of p16INK4A, a new marker that correlates with dysplasia in oral squamoproliferative lesions. *Am J Surg Pathol* **29:** 792–796.

Gonzalez MV, Pello MF, Lopez-Larrea C, Suarez C, Menendez MJ, Coto E (1995). Loss of heterozygosity and mutation analysis of the p16 (9p21) and p53 (17p13) genes in squamous cell carcinoma of the head and neck. *Clin Cancer Res* **1:** 1043–1049.

Gonzalez-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I (2000). Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. *Head Neck* **22:** 658–661.

Grandis JR, Tweardy DJ (1993). Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res* **53:** 3579–3584.

Guo Z, Yamaguchi K, Sanchez-Cespedes M, Westra WH, Koch WM, Sidransky D (2001). Allelic losses in OraTest-directed biopsies of patients with prior upper aerodigestive tract malignancy. *Clin Cancer Res* **7:** 1963–1968.

Gutkind JS (1998). The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* **273:** 1839–1842.

Ha PK, Califano JA (2006). Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. *Lancet Oncol* **7:** 77–82.

Ha PK, Pilkington TA, Westra WH, Sciubba J, Sidransky D, Califano JA (2002). Progression of microsatellite instability from premalignant lesions to tumors of the head and neck. *Int J Cancer* **102:** 615–617.

Hasina R, Whipple ME, Martin LE, Kuo WP, Ohno-Machado L, Lingen MW (2008). Angiogenic heterogeneity in head and neck squamous cell carcinoma: biological and therapeutic implications. *Lab Invest* **88:** 342–353.

Hemmer J, Nagel E, Kraft K (1999). DNA aneuploidy by flow cytometry is an independent prognostic factor in squamous cell carcinoma of the oral cavity. *Anticancer Res* **19:** 1419–1422.

Holmstrup P, Vedtofte P, Reibel J, Stoltze K (2006). Long-term treatment outcome of oral premalignant lesions. *Oral Oncol* **42:** 461–474.

Huang M, Spitz MR, Gu J *et al* (2006). Cyclin D1 gene polymorphism as a risk factor for oral premalignant lesions. *Carcinogenesis* **27:** 2034–2037.

Iamaroon A, Khemaleelakul U, Pongsiriwet S, Pintong J (2004). Co-expression of p53 and Ki67 and lack of EBV expression in oral squamous cell carcinoma. *J Oral Pathol Med* **33:** 30–36.

Inoue K, Ozeki Y, Suganuma T, Sugiura Y, Tanaka S (1997). Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma. Association with angiogenesis and tumor progression. *Cancer* **79:** 206–213.

Ishida K, Ito S, Wada N *et al* (2007). Nuclear localization of beta-catenin involved in precancerous change in oral leukoplakia. *Mol Cancer* **6:** 62.

Islam MN, Kornberg L, Veenker E, Cohen DM, Bhattacharyya I (2010). Anatomic site based ploidy analysis of oral premalignant lesions. *Head Neck Pathol* **4:** 10–14.

Jiang WW, Fujii H, Shirai T, Mega H, Takagi M (2001). Accumulative increase of loss of heterozygosity from leukoplakia to foci of early cancerization in leukoplakia of the oral cavity. *Cancer* **92:** 2349–2356.

Johnstone S, Logan RM (2006). The role of vascular endothelial growth factor (VEGF) in oral dysplasia and oral squamous cell carcinoma. *Oral Oncol* **42:** 337–342.

Johnstone S, Logan RM (2007). Expression of vascular endothelial growth factor (VEGF) in normal oral mucosa, oral dysplasia and oral squamous cell carcinoma. *Int J Oral Maxillofac Surg* **36:** 263–266.

Jordan RC, Bradley G, Slingerland J (1998). Reduced levels of the cell-cycle inhibitor p27Kip1 in epithelial dysplasia and carcinoma of the oral cavity. *Am J Pathol* **152:** 585–590.

Kahn MA, Dockter ME, Hermann-Petrin JM (1994). Proliferative verrucous leukoplakia. Four cases with flow cytometric analysis. *Oral Surg Oral Med Oral Pathol* **78:** 469–475.

Kato K, Hara A, Kuno T *et al* (2006). Aberrant promoter hypermethylation of p16 and MGMT genes in oral squamous cell carcinomas and the surrounding normal mucosa. *J Cancer Res Clin Oncol* **132:** 735–743.

Kaur J, Sawhney M, Dattagupta S, Shukla NK, Srivastava A, Ralhan R (2010). Clinical significance of phosphatidyl inositol synthase overexpression in oral cancer. *BMC Cancer* **10:** 168.

Kayahara H, Yamagata H, Tanioka H, Miki T, Hamakawa H (2001). Frequent loss of heterozygosity at 3p25-p26 is associated with invasive oral squamous cell carcinoma. *J Hum Genet* **46:** 335–341.

Kim HR, Christensen R, Park NH, Sapp P, Kang MK (2001). Elevated expression of hTERT is associated with dysplastic cell transformation during human oral carcinogenesis in situ. *Clin Cancer Res* **7:** 3079–3086.

Klanrit P, Sperandio M, Brown AL *et al* (2007). DNA ploidy in proliferative verrucous leukoplakia. *Oral Oncol* **43:** 310–316.

Kodani I, Shomori K, Osaki M, Kuratate I, Ryoke K, Ito H (2001). Expression of minichromosome maintenance 2 (MCM2), Ki-67, and cell-cycle-related molecules, and apoptosis in the normal-dysplasia-carcinoma sequence of the oral mucosa. *Pathobiology* **69:** 150–158.

Kodani I, Osaki M, Shomori K *et al* (2003). Minichromosome maintenance 2 expression is correlated with mode of invasion and prognosis in oral squamous cell carcinomas. *J Oral Pathol Med* **32:** 468–474.

Kovesi G, Szende B (2006). Prognostic value of cyclin D1, p27, and p63 in oral leukoplakia. *J Oral Pathol Med* **35:** 274–277.

Kresty LA, Mallery SR, Knobloch TJ *et al* (2002). Alterations of p16(INK4a) and p14(ARF) in patients with severe oral epithelial dysplasia. *Cancer Res* **62:** 5295–5300.

Kudo Y, Takata T, Ogawa I *et al* (2000a). p27Kip1 accumulation by inhibition of proteasome function induces apoptosis in oral squamous cell carcinoma cells. *Clin Cancer Res* **6:** 916–923.

Kudo Y, Takata T, Ogawa I *et al* (2000b). Reduced expression of p27(Kip1) correlates with an early stage of cancer invasion in oral squamous cell carcinoma. *Cancer Lett* **151:** 217–222.

Kulkarni V, Saranath D (2004). Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. *Oral Oncol* **40:** 145–153.

Kuo MY, Hsu HY, Kok SH *et al* (2002). Prognostic role of p27(Kip1) expression in oral squamous cell carcinoma in Taiwan. *Oral Oncol* **38:** 172–178.

Kushner J, Bradley G, Jordan RC (1997). Patterns of p53 and Ki-67 protein expression in epithelial dysplasia from the floor of the mouth. *J Pathol* **183:** 418–423.

Lai S, El-Naggar AK (1999). Differential expression of key cell cycle genes (p16/cyclin D1/pRb) in head and neck squamous carcinomas. *Lab Invest* **79:** 255–260.

Lee J, Kim SS (2009). The function of p27 KIP1 during tumor development. *Exp Mol Med* **41:** 765–771.

Lee JJ, Hong WK, Hittelman WN *et al* (2000). Predicting cancer development in oral leukoplakia: ten years of translational research. *Clin Cancer Res* **6:** 1702–1710.

Li C, Shintani S, Terakado N *et al* (2005). Microvessel density and expression of vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived endothelial growth factor in oral squamous cell carcinomas. *Int J Oral Maxillofac Surg* **34:** 559–565.

Li JN, Feng CJ, Lu YJ *et al* (2008). mRNA expression of the DNA replication-initiation proteins in epithelial dysplasia and squamous cell carcinoma of the tongue. *BMC Cancer* **8:** 395.

Liao J, Mitsuyasu T, Yamane K, Ohishi M (2000). Telomerase activity in oral and maxillofacial tumors. *Oral Oncol* **36:** 347–352.

Loro LL, Johannessen AC, Vintermyr OK (2002). Decreased expression of bcl-2 in moderate and severe oral epithelia dysplasias. *Oral Oncol* **38:** 691–698.

Macluskey M, Chandrachud LM, Pazouki S *et al* (2000). Apoptosis, proliferation, and angiogenesis in oral tissues. Possible relevance to tumour progression. *J Pathol* **191:** 368–375.

Mao L, Lee JS, Fan YH *et al* (1996). Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. *Nat Med* **2:** 682–685.

Marshall CJ (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80:** 179–185.

Massarelli E, Liu DD, Lee JJ *et al* (2005). Akt activation correlates with adverse outcome in tongue cancer. *Cancer* **104:** 2430–2436.

Mehanna HM, Rattay T, Smith J, McConkey CC (2009). Treatment and follow-up of oral dysplasia – a systematic review and meta-analysis. *Head Neck* **31:** 1600–1609.

Mineta H, Miura K, Takebayashi S *et al* (2000). Cyclin D1 overexpression correlates with poor prognosis in patients with tongue squamous cell carcinoma. *Oral Oncol* **36:** 194–198.

Mishima K, Inoue K, Hayashi Y (2002). Overexpression of extracellular-signal regulated kinases on oral squamous cell carcinoma. *Oral Oncol* **38:** 468–474.

Miyoshi Y, Tsukinoki K, Imaizumi T *et al* (1999). Telomerase activity in oral cancer. *Oral Oncol* **35:** 283–289.

Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS (2009). Dysregulated molecular networks in head and neck carcinogenesis. *Oral Oncol* **45:** 324–334.

Moriyama M, Kumagai S, Kawashiri S, Kojima K, Kakihara K, Yamamoto E (1997). Immunohistochemical study of tumour angiogenesis in oral squamous cell carcinoma. *Oral Oncol* **33:** 369–374.

Muirhead DM, Hoffman HT, Robinson RA (2006). Correlation of clinicopathological features with immunohistochemical expression of cell cycle regulatory proteins p16 and retinoblastoma: distinct association with keratinisation and differentiation in oral cavity squamous cell carcinoma. *J Clin Pathol* **59:** 711–715.

Murti PR, Warnakulasuriya KA, Johnson NW *et al* (1998). p53 expression in oral precancer as a marker for malignant potential. *J Oral Pathol Med* **27:** 191–196.

Mutirangura A, Supiyaphun P, Trirekapan S *et al* (1996). Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. *Cancer Res* **56:** 3530–3533.

Nagatsuka H, Ishiwari Y, Tsujigiwa H, Nakano K, Nagai N (2001). Quantitation of epidermal growth factor receptor gene amplification by competitive polymerase chain reaction in pre-malignant and malignant oral epithelial lesions. *Oral Oncol* **37:** 599–604.

el-Naggar AK, Hurr K, Batsakis JG, Luna MA, Goepfert H, Huff V (1995). Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous carcinoma. *Cancer Res* **55:** 2656–2659.

Nagpal JK, Patnaik S, Das BR (2002). Prevalence of high-risk human papilloma virus types and its association with P53 codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of Eastern India. *Int J Cancer* **97:** 649–653.

Nakahara Y, Shintani S, Mihara M, Kiyota A, Ueyama Y, Matsumura T (2000). Alterations of Rb, p16(INK4A) and cyclin D1 in the tumorigenesis of oral squamous cell carcinomas. *Cancer Lett* **160:** 3–8.

Nemes JA, Nemes Z, Marton IJ (2005). p21WAF1/CIP1 expression is a marker of poor prognosis in oral squamous cell carcinoma. *J Oral Pathol Med* **34:** 274–279.

Oliveira LR, Ribeiro-Silva A, Costa JP, Simoes AL, Matteo MA, Zucoloto S (2008). Prognostic factors and survival analysis in a sample of oral squamous cell carcinoma patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **106:** 685–695.

Olshan AF, Weissler MC, Pei H, Conway K (1997). p53 mutations in head and neck cancer: new data and evaluation of mutational spectra. *Cancer Epidemiol Biomarkers Prev* **6:** 499–504.

Pande P, Mathur M, Shukla NK, Ralhan R (1998). pRb and p16 protein alterations in human oral tumorigenesis. *Oral Oncol* **34:** 396–403.

Pannone G, De Maria S, Zamparese R *et al* (2007). Prognostic value of human telomerase reverse transcriptase gene expression in oral carcinogenesis. *Int J Oncol* **30:** 1349–1357.

Papadimitrakopoulou V, Izzo J, Lippman SM *et al* (1997). Frequent inactivation of p16INK4a in oral premalignant lesions. *Oncogene* **14:** 1799–1803.

Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J (1997). Field cancerisation of the oral cavity: comparison of the spectrum of molecular alterations in cases presenting with both dysplastic and malignant lesions. *Oral Oncol* **33:** 332–337.

Partridge M, Emilion G, Pateromichelakis S, A'Hern R, Phillips E, Langdon J (1998). Allelic imbalance at chromosomal loci implicated in the pathogenesis of oral precancer, cumulative loss and its relationship with progression to cancer. *Oral Oncol* **34:** 77–83.

Partridge M, Pateromichelakis S, Phillips E, Emilion GG, A'Hern RP, Langdon JD (2000). A case–control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization. *Cancer Res* **60:** 3893–3898.

Partridge M, Pateromichelakis S, Phillips E, Emilion G, Langdon J (2001). Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma. *Clin Cancer Res* **7:** 1860–1866.

Pazouki S, Chisholm DM, Adi MM *et al* (1997). The association between tumour progression and vascularity in the oral mucosa. *J Pathol* **183:** 39–43.

Pentenero M, Giaretti W, Navone R *et al* (2009). DNA aneuploidy and dysplasia in oral potentially malignant disorders: association with cigarette smoking and site. *Oral Oncol* **45:** 887–890.

Poeta ML, Manola J, Goldwasser MA *et al* (2007). TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *N Engl J Med* **357:** 2552–2561.

Poh CF, Zhang L, Lam WL *et al* (2001). A high frequency of allelic loss in oral verrucous lesions may explain malignant risk. *Lab Invest* **81:** 629–634.

Reed AL, Califano J, Cairns P *et al* (1996). High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. *Cancer Res* **56:** 3630–3633.

Ries LAGMD, Krapcho M, Stinchcomb DG *et al* (eds) (2008). SEER Cancer Statistics Review, 1975–2005. Bethesda, MD: National Cancer Institute.

Rosin MP, Epstein JB, Berean K *et al* (1997). The use of exfoliative cell samples to map clonal genetic alterations in the oral epithelium of high-risk patients. *Cancer Res* **57:** 5258–5260.

Rosin MP, Cheng X, Poh C *et al* (2000). Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. *Clin Cancer Res* **6:** 357–362.

Rosin MP, Lam WL, Poh C *et al* (2002). 3p14 and 9p21 loss is a simple tool for predicting second oral malignancy at previously treated oral cancer sites. *Cancer Res* **62:** 6447–6450.

Roz L, Wu CL, Porter S *et al* (1996). Allelic imbalance on chromosome 3p in oral dysplastic lesions: an early event in oral carcinogenesis. *Cancer Res* **56:** 1228–1231.

Rubio Bueno P, Naval Gias L, Garcia Delgado R, Domingo Cebollada J, Diaz Gonzalez FJ (1998). Tumor DNA content as a prognostic indicator in squamous cell carcinoma of the oral cavity and tongue base. *Head Neck* **20:** 232–239.

Saito T, Yamashita T, Notani K *et al* (1995). Flow cytometric analysis of nuclear DNA content in oral leukoplakia: relation to clinicopathologic findings. *Int J Oral Maxillofac Surg* **24:** 44–47.

Schlecht NF (2005). Prognostic value of human papillomavirus in the survival of head and neck cancer patients: an overview of the evidence. *Oncol Rep* **14:** 1239–1247.

Schoelch ML, Le QT, Silverman S Jr *et al* (1999a). Apoptosis-associated proteins and the development of oral squamous cell carcinoma. *Oral Oncol* **35:** 77–85.

Schoelch ML, Regezi JA, Dekker NP *et al* (1999b). Cell cycle proteins and the development of oral squamous cell carcinoma. *Oral Oncol* **35:** 333–342.

Seoane J, Bascones A, Asenjo JA, Garcia-Pola M, Varela-Centelles PI (1998). Flow cytometric analysis of nuclear DNA content in oral leukoplakia. *Clin Otolaryngol Allied Sci* **23:** 136–140.

Shah NG, Trivedi TI, Tankshali RA *et al* (2007). Molecular alterations in oral carcinogenesis: significant risk predictors in malignant transformation and tumor progression. *Int J Biol Markers* **22:** 132–143.

Shay JW, Wright WE (2010). Telomeres and telomerase in normal and cancer stem cells. *FEBS Lett* **584:** 3819–3825.

Shin DM, Ro JY, Hong WK, Hittelman WN (1994). Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. *Cancer Res* **54:** 3153–3159.

Shin DM, Lee JS, Lippman SM *et al* (1996). p53 expressions: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. *J Natl Cancer Inst* **88:** 519–529.

Shintani S, Mihara M, Nakahara Y *et al* (2002). Expression of cell cycle control proteins in normal epithelium, premalignant and malignant lesions of oral cavity. *Oral Oncol* **38:** 235–243.

Shintani S, Li C, Mihara M, Hino S, Nakashiro K, Hamakawa H (2003). Skp2 and Jab1 expression are associated with inverse expression of p27(KIP1) and poor prognosis in oral squamous cell carcinomas. *Oncology* **65:** 355–362.

Shintani S, Li C, Ishikawa T, Mihara M, Nakashiro K, Hamakawa H (2004). Expression of vascular endothelial growth factor A, B, C, and D in oral squamous cell carcinoma. *Oral Oncol* **40:** 13–20.

Smeenk L, Lohrum M (2010). Behind the scenes: unravelling the molecular mechanisms of p53 target gene selectivity (Review). *Int J Oncol* **37:** 1061–1070.

Smith J, Rattay T, McConkey C, Helliwell T, Mehanna H (2009). Biomarkers in dysplasia of the oral cavity: a systematic review. *Oral Oncol* **45:** 647–653.

Soni S, Kaur J, Kumar A *et al* (2005). Alterations of rb pathway components are frequent events in patients with oral epithelial dysplasia and predict clinical outcome in patients with squamous cell carcinoma. *Oncology* **68:** 314–325.

Soria JC, Morat L, Commo F *et al* (2001). Telomerase activation cooperates with inactivation of p16 in early head and neck tumorigenesis. *Br J Cancer* **84:** 504–511.

Srinivasan M, Jewell SD (2001). Evaluation of TGF-alpha and EGFR expression in oral leukoplakia and oral submucous fibrosis by quantitative immunohistochemistry. *Oncology* **61:** 284–292.

Sumida T, Sogawa K, Hamakawa H, Sugita A, Tanioka H, Ueda N (1998). Detection of telomerase activity in oral lesions. *J Oral Pathol Med* **27:** 111–115.

Suzuki H, Sugimura H, Hashimoto K (2006). p16INK4A in oral squamous cell carcinomas – a correlation with biological behaviors: immunohistochemical and FISH analysis. *J Oral Maxillofac Surg* **64:** 1617–1623.

Szelachowska J, Dziegiel P, Jelen-Krzeszewska J *et al* (2006). Mcm-2 protein expression predicts prognosis better than Ki-67 antigen in oral cavity squamocellular carcinoma. *Anticancer Res* **26:** 2473–2478.

Tabor MP, Brakenhoff RH, van Houten VM *et al* (2001). Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res* **7:** 1523–1532.

Tabor MP, Braakhuis BJ, van der Wal JE *et al* (2003). Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx. *J Pathol* **199:** 354–360.

Tae K, El-Naggar AK, Yoo E *et al* (2000). Expression of vascular endothelial growth factor and microvessel density in head and neck tumorigenesis. *Clin Cancer Res* **6:** 2821–2828.

Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI, Semba I (2006). Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. *J Oral Pathol Med* **35:** 369–375.

Takeshima M, Saitoh M, Kusano K *et al* (2008). High frequency of hypermethylation of p14, p15 and p16 in oral pre-cancerous lesions associated with betel-quid chewing in Sri Lanka. *J Oral Pathol Med* **37:** 475–479.

Taoudi Benchekroun M, Saintigny P, Thomas SM *et al* (2010). Epidermal growth factor receptor expression and gene copy number in the risk of oral cancer. *Cancer Prev Res (Phila)* **3:** 800–809.

Temam S, Flahault A, Perie S *et al* (2000). p53 gene status as a predictor of tumor response to induction chemotherapy of patients with locoregionally advanced squamous cell carcinomas of the head and neck. *J Clin Oncol* **18:** 385–394.

Thurfjell N, Coates PJ, Boldrup L *et al* (2005). Function and importance of p63 in normal oral mucosa and squamous cell carcinoma of the head and neck. *Adv Otorhinolaryngol* **62:** 49–57.

Todd R, Hinds PW, Munger K *et al* (2002). Cell cycle dysregulation in oral cancer. *Crit Rev Oral Biol Med* **13:** 51–61.

Torres-Rendon A, Roy S, Craig GT, Speight PM (2009a). Expression of Mcm2, geminin and Ki67 in normal oral mucosa, oral epithelial dysplasias and their corresponding squamous-cell carcinomas. *Br J Cancer* **100:** 1128–1134.

Torres-Rendon A, Stewart R, Craig GT, Wells M, Speight PM (2009b). DNA ploidy analysis by image cytometry helps to identify oral epithelial dysplasias with a high risk of malignant progression. *Oral Oncol* **45:** 468–473.

Tsui IF, Rosin MP, Zhang L, Ng RT, Lam WL (2008). Multiple aberrations of chromosome 3p detected in oral premalignant lesions. *Cancer Prev Res (Phila)* **1:** 424–429.

Tsui IF, Poh CF, Garnis C, Rosin MP, Zhang L, Lam WL (2009). Multiple pathways in the FGF signaling network are frequently deregulated by gene amplification in oral dysplasias. *Int J Cancer* **125:** 2219–2228.

Turatti E, da Costa Neves A, de Magalhaes MH, de Sousa SO (2005). Assessment of c-Jun, c-Fos and cyclin D1 in premalignant and malignant oral lesions. *J Oral Sci* **47:** 71–76.

Venkatesan TK, Kuropkat C, Caldarelli DD *et al* (1999). Prognostic significance of p27 expression in carcinoma of the oral cavity and oropharynx. *Laryngoscope* **109:** 1329–1333.

Vered M, Allon I, Dayan D (2009). Maspin, p53, p63, and Ki-67 in epithelial lesions of the tongue: from hyperplasia through dysplasia to carcinoma. *J Oral Pathol Med* **38:** 314–320.

Vousden KH, Lane DP (2007). p53 in health and disease. *Nat Rev Mol Cell Biol* **8:** 275–283.

Warnakulasuriya S, Jia C, Johnson N, Houghton J (2000). p53 and P-glycoprotein expression are significant prognostic markers in advanced head and neck cancer treated with chemo/radiotherapy. *J Pathol* **191:** 33–38.

Warnakulasuriya S, Reibel J, Bouquot J, Dabelsteen E (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *J Oral Pathol Med* **37:** 127–133.

Watanabe S, Sato K, Okazaki Y, Tonogi M, Tanaka Y, Yamane GY (2009). Activation of PI3K-AKT pathway in oral epithelial dysplasia and early cancer of tongue. *Bull Tokyo Dent Coll* **50:** 125–133.

Watanabe S, Watanabe R, Oton-Leite AF *et al* (2010). Analysis of cell proliferation and pattern of invasion in oral squamous cell carcinoma. *J Oral Sci* **52:** 417–424.

Wong TS, Man MW, Lam AK, Wei WI, Kwong YL, Yuen AP (2003). The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR. *Eur J Cancer* **39:** 1881–1887.

Xie X, De Angelis P, Clausen OP, Boysen M (1999). Prognostic significance of proliferative and apoptotic markers in oral tongue squamous cell carcinomas. *Oral Oncol* **35:** 502–509.

Xie X, Clausen OP, Boysen M (2002). Prognostic significance of p21WAF1/CIP1 expression in tongue squamous cell carcinomas. *Arch Otolaryngol Head Neck Surg* **128:** 897–902.

Xu J, Gimenez-Conti IB, Cunningham JE *et al* (1998). Alterations of p53, cyclin D1, Rb, and H-ras in human oral carcinomas related to tobacco use. *Cancer* **83:** 204–212.

Yajima Y, Noma H, Furuya Y *et al* (2004). Quantification of telomerase activity of regions unstained with iodine solution that surround oral squamous cell carcinoma. *Oral Oncol* **40:** 314–320.

Ye Y, Lippman SM, Lee JJ *et al* (2008). Genetic variations in cell-cycle pathway and the risk of oral premalignant lesions. *Cancer* **113:** 2488–2495.

Yoo GH, Xu HJ, Brennan JA *et al* (1994). Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma. *Cancer Res* **54:** 4603–4606.

Youssef EM, Lotan D, Issa JP *et al* (2004). Hypermethylation of the retinoic acid receptor-beta(2) gene in head and neck carcinogenesis. *Clin Cancer Res* **10:** 1733–1742.

Zhang L, Zhang W (1999). Telomerase hTR and hTRT gene expression in oral precancerous lesions and squamous cell carcinomas. *Chin J Dent Res* **2:** 43–48.

Zhang L, Cheung KJ Jr, Lam WL *et al* (2001a). Increased genetic damage in oral leukoplakia from high risk sites: potential impact on staging and clinical management. *Cancer* **91:** 2148–2155.

Zhang L, Poh CF, Lam WL *et al* (2001b). Impact of localized treatment in reducing risk of progression of low-grade oral dysplasia: molecular evidence of incomplete resection. *Oral Oncol* **37:** 505–512.

Zhang L, Williams M, Poh CF *et al* (2005). Toluidine blue staining identifies high-risk primary oral premalignant lesions with poor outcome. *Cancer Res* **65:** 8017–8021.