Loss of heterozygosity as a marker to predict progression of oral epithelial dysplasia to oral squamous cell carcinoma

Head and neck squamous cell carcinoma is the sixth most common malignancy, with an annual incidence of 300,000 new cases[1-3] diagnosed worldwide, with particularly high incidence rates in South and Southeast Asia, Europe, Latin America and the Caribbean and Pacific nations. Globally varying studies have been addressing the fundamental aspects of this malignancy with respect to its prevention, early diagnosis and management.[1-4] Although the effort taken is phenomenal, the morbidity and mortality associated with oral squamous cell carcinoma (OSCC) is discouraging.[5] The survival rate is directly proportional to the stage of the disease at the time of diagnosis. It is 80% for Stage I cancers but drops to 20% for Stage IV cancers.[6] Unfavorable outcome due to the disease is further burdened by the morbidities, accompanying deformities due to surgery and those seen after radiation as complications, namely, mucositis and osteoradionecrosis, which have a deleterious impact on the quality of life of the affected individual.[3]

The term oral potentially malignant disorders (OPMDs) was recommended at the World Health Organization (WHO) workshop held in 2005.[7] An oral premalignant lesion is defined as any lesion or condition of the oral mucosa that has the potential for malignant transformation (MT). This encompasses a number of oral lesions, such as leukoplakia, erythroplakia, erythroleukoplakia, erosive lichen planus, oral submucous fibrosis and oral dysplasia. OPMDs are a spectrum of lesions and conditions of the oral mucosa, which are characterized by an increased risk of MT to OSCC of which leukoplakia and erythroplakia are the most common OPMDs.[8] Leukoplakia is defined by the WHO as a "white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer."[9] Leukoplakia is a clinical terminology.[10] Histopathologically, this lesion may be exhibiting atrophy, hyperplasia or dysplasia. It has been established that OPMDs[11] could be the precursor lesions of OSCC.[12] These lesions are predominantly associated with habits, namely, chronic use of tobacco and excess consumption of alcohol.[13] OPMDs are also described as a group of disorders of varying etiologies, characterized by mutagen associated, spontaneous or hereditary alterations or mutations in the genetic material of oral epithelial cells with or without clinical and histopathological alterations that may transform to OSCC.[14] Of the OPMDs, oral leukoplakia is the most prevalent one with a prevalence ranging from 0.4% to 2.6% of the population worldwide, with a MT rate between 3.0% and 17.5%.[8,15,16]

Preferred and accepted marker to assess the risk of an OPMD eventually undergoing MT is the presence and grade of dysplasia in the lesion. Dysplasia is defined as the presence of specific epithelial architectural and cytologic changes and is graded as mild, moderate, or severe based on the depth and severity of the changes. It is frequently assumed that oral carcinogenesis involves OPMDs that undergo a gradual progression beginning with hyperplasia and evolving through stages of mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma *in situ* and finally carcinoma after cellular invasion through the basement membrane. Till today, the pronounced and accountable predictor of MT in a mucosal lesion is epithelial dysplasia which is described by WHO as a spectrum of architectural and cytological epithelial changes caused by accumulation of genetic changes, associated with risk of progression to OSCC. The WHO has now introduced the binary system of dysplasia grading into high grade and low grade, to address and overcome the challenges and limitations of the three grading systems.[17] The truth, sometimes, is that the progress of dysplasia to cancer does not necessarily occur

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in a systematic way and dysplasia in all OPMDs does not progress to OSCC.

The observational studies have reported that with increasing grade of dysplasia, the MT rate increases and estimated the transformation rate as 12.1% (confidence interval [CI]: 8.1%–17.9%) for dysplastic lesions.[18] Analytical studies assessing the risk of transition from OPMDs to cancer have identified clinicopathological parameters that may be associated with an increased risk of MT.[6,19] Since the MT of potentially premalignant oral epithelial conditions cannot be predicted exclusively on the basis of clinical features, histological assessment of the biopsied lesions is necessary, but the limitations which accompany these findings are variability and lack of definitive criteria in the interpretation of dysplastic changes. Thus, biopsy of a suspected lesion can ascertain its malignant potential but lacks the power to predict the outcome of an initiated malignant change. The two issues of concern in this context are the fact that progression to OSCC is not a defined linear event, and at the same time, the dysplastic lesion may not progress to malignancy. On the other hand, histologically normal appearing lesion may be the forerunner of molecular premalignant lesion which is yet to develop morphological/cytological changes consistent with dysplasia.[4,20] Holmstrup *et al*. in their study of long-term outcome of oral premalignant lesions concluded that dysplasia grading did not have any influence on the risk of MT.[21]

Lingen *et al*. in his review on genetics/epigenetics of oral premalignancy explained that cancer is driven by the accumulation of genetic and epigenetic changes within a clonal population of cells.[4] The resulting genotypic alterations can affect numerous genes and this could be followed by phenotypic changes affecting critical cellular functions such as resistance to cell death, increased proliferation, induction of angiogenesis and ability to metastasize. The epigenetic changes which can affect the gene expression include DNA methylation, histone acetylation and expression of small noncoding RNAs. Global methylation in head and neck squamous cell carcinomas has been associated with poor prognosis, but conversely certain epigenetic involvement has therapeutic implications.[22]

Human papillomavirus (HPV) has been implicated in the etiology of head and neck squamous cell carcinomas in about 20%–30% of all new cases, especially those affecting the oropharyngeal region.[23,24] Based on HPV involvement, head and neck squamous cell carcinomas are categorized into two genetic subclasses, namely, HPV-positive and HPV-negative tumors. HPV-positive tumors are seen more in the younger age group and do not necessarily have the risk factors of smoking and excessive alcohol use and have a favorable clinical outcome compared to HPV-negative neoplasms.[5] This documented biological behavior outcome of HPV-positive tumors was adopted to tumor-node-metastasis staging for head and neck squamous cell carcinomas in the eight edition.[5,25] Over the past decade, studies on the role of HPV have also identified genetically distinct HPV-negative head and neck tumors with favorable prognosis.[5]

Parallel to the studies trying to understand the pathogenesis of OSCC and those trying to address the concept of premalignancy, there have also been commendable advances in recent years with respect to detection, prevention and management of OSCC. In spite of the advances, the morbidity and mortality associated with this lesion is not encouraging. One of the probable reasons for this could be late diagnosis and "field cancerization" resulting in the development of multiple primary tumors which have a negative impact on survival rate.[26]

Early detection of both OPMD and OSCC is essential to prevent the progression of the disease to a devastating one and also to reduce the morbidity and mortality. In the recent past, diagnostic adjuncts from a clinical perspective, namely, light-based handheld devices and cytology with or additional analyses, *in vivo* imaging with molecular probes/paints and salivary diagnostics are being used effectively. The efficacy of the adjunct would be directly attributed to identification of biomarkers, which could predict as to which oral mucosal potential premalignant disorder is likely to progress to malignancy. Thus, the fundamental need would be to identify these lesions and manage them successfully and thus prevent MT.[3,10,27] The clinical diagnosis of leukoplakia includes lesions carrying a risk for cancer progression. Till today, histopathological evaluation retains its value for assessing the risk involved in MT but with the limitations of predicting MT of those cases with no dysplasia or minimal dysplasia. Diagnosing cytological and morphological alterations as a feature of oral epithelial dysplasia (OED) is essential to predict MT, but the caution would be that the absence of microscopic dysplasia should not eliminate the possibility of an underlying potential malignant disorder. Molecular biomarkers have a role to distinguish and stratify the oral premalignancy candidates into low- and high risk categories for progression to OSCC. Their appropriate application could dramatically improve our ability to diagnose and treat oral premalignancy. Advances in the knowledge of carcinogenesis have contributed significantly to the

knowledge of potential biomarkers which could reflect the premalignant nature of the lesion in question with predictive ability.[24,26]

One such important biomarker is loss of heterozygosity (LOH). LOH occurs in a somatic cell as a result of loss of genomic material specifically affecting the single retained copy of a fundamental allele. Microsatellite analysis for LOH is used to assess the loss of chromosomal regions that contain known or putative tumor suppressor genes.[28-30]

Mao *et al*. in their ongoing chemoprevention trial studied 84 leukoplakia samples from 37 patients for two microsatellite markers located at chromosomes 9p21 and 3p14. Their results showed that 51% patients (19/37) of their study group showed LOH on either or both loci.[29] Of these 19 patients, 8 patients developed OSCC. Of these 8, 7 patients had LOH. Based on their findings, they postulated that clonal genetic alterations are a feature of oral premalignant lesions. This finding was further supported by Zhang and Rosin, who also confirmed that LOH (loss of specific chromosomal regions) which contains known or presumptive tumor suppressor genes could be an early predictor of subsequent progression of oral premalignant lesions.[30]

To further consider if LOH could be used as a biomarker, we need to know if LOH was associated with features of dysplasia, grades of dysplasia and its utility to predict individuals as low- or high risk candidates for MT, especially in the context of precession medicine therapy. We also need to know if this prediction can be applicable to the developing new lesions in the purview of field cancerization.[28,31]

Rosin *et al*. studied LOH in cases of early oral premalignancies and compared these findings with LOH profiles of those cases with a history of progression to carcinoma *in situ* or invasive carcinoma and those without a history of progression, referred to as nonprogressing cases.[32] The criteria used for nonprogressing cases were those cases with a histological diagnosis of hyperplasia or mild or moderate dysplasia and also had no subsequent history of head and neck cancer. The progressing cases had a histological diagnosis of hyperplasia or low-grade dysplasia and later progressed to carcinoma *in situ* or OSCC. They totally studied 116 cases which were analyzed for LOH at 19 microsatellite loci on 7 chromosomes arms (3p, 4q, 8p, 9q, 11q, 13q and 17p). Their study showed that progressing and nonprogressing cases had significant differences in their LOH profiles, which supports the hypothesis that LOH patterns could be considered as a biomarker of MT. Individuals with LOH at 3p and/or 9p but at no other arms showed increase of 3.8-fold in relative risk for developing cancer. In contrast, individuals with accompanying additional losses on 4p, 8p, 11q or 17p, as seen in nonprogressing cases showed 33-fold in increase in relative cancer risk. Their studies reconfirmed the findings of Mao *et al*., who found an association of LOH at 9p21 and 3p14 positions and progression of premalignant lesions.[29] The authors also stressed the need for studies to include LOH profile at additional sites, since these results could reflect the time taken by the dysplastic lesion to progress into cancer. They also stated that there was a greater than 20-fold increase in progression risk for lesions with 3p and/or 9p LOH compared with lesions with retention of these two regions. These authors further validated their above preliminary findings of LOH profiles as risk predictors using a community-based new prospective cohort enrolled in a longitudinal study of low-grade oral premalignant lesions from a population-based patient group.[33] This prospective cohort included 296 patients with a histological diagnosis of primary mild/moderate dysplasia, and patients were classified into high- or low risk profiles, in order to validate their previous 2000 model. The results of their prospective study showed that that the high risk lesions which exhibited 3p and/or 9p LOH had a 22.6-fold increase in risk compared with low risk lesions which had 3p and 9p retention. When further analysis was done with addition of another two markers (loci on 4q/17p), the risk prediction was further enhanced, with 5-year progression rates of 3.1%, 16.3% and 63.1% for the low risk (9p retention), intermediate risk (9p LOH or 9p LOH with either 17p LOH or 4q LOH but not both) and high risk lesions (9p LOH with both 17p LOH and 4q LOH), respectively.

They also highlighted the importance of 9p21 in this prospective study as a predictor of progression of oral premalignant epithelial conditions than 3p14 and stated that LOH on 3p may represent a passenger alteration rather than a driving force for progression. Graveland *et al*. studied exfoliated cells and biopsied tissue of 43 patients with leukoplakia (6 of these cases progressed to oral cancer) to study LOH profiles at chromosomes 3p, 9p, 11q and 17p and also performed additional analysis of immunohistochemical staining of biopsied tissue for p53 and TP53 mutation analysis.[34] They showed that LOH was present in 51% of cases at 9p. These results also confirmed that mutated TP53 and LOH at 9p in the biopsy as individual markers and in combination were significant predictors of malignant progression of leukoplakia to oral cancer.

Fonseca-Silva *et al*. assessed the histological parameters which are used to grade dysplasia with the LOH profile.[35] The different grades of dysplasia did not show differences in the frequencies of LOH, but their study highlighted that histological features of dysplasia were associated with specific LOH. Irregular stratification was associated with LOH at marker D3S1234 (3p14.2); drop-shaped rete ridges and premature keratinization in single cells showed associations with LOH at D9S162A and p53 (17p13.1), respectively. Based on these findings, they indicate that each architecture abnormality could probably be a molecular signature profile, and these features of dysplasia could be the result of different molecular alterations communicating different biological meanings, which need to be further explored.[36]

Sufficient literature evidence has been documented to strongly support the association between different forms of tobacco exposure and an increased risk of OSCC, and there are many observational and prospective studies that have also described that tobacco usage positively influences the risk of potentially premalignant oral epithelial lesions. This thus mandates that smokers should be screened to detect early oral potentially malignant lesions. An added observation is that smoking cessation would result in beneficial results to a population by reducing oral leukoplakia prevalence and oral cancer incidence.[11,37] However, OSCC does develop in nonsmokers and there are indications that oral potentially malignant lesions in nonsmokers possess a higher cancer risk than those in smokers.[20,33,38,39] Without tobacco as an etiology, the development of OPMDs in nonsmokers may suggest genetic susceptibility.

Rock *et al*., in order to understand the incidence of OSCC in nonsmokers, wanted to ascertain the natural history of OED in nonsmokers as compared to smokers in their population-based cohort with >10-year follow-up. The strength of the study was the complete documentation of all the variables associated with demographics, detailed habit history, clinical information and inclusion of results obtained by toluidine blue and fluorescent visualization. Their studies also included microsatellite analysis for LOH based on their previous experience that LOH markers can delineate high risk lesions, regardless of risk habits, and thus may be important in strategic evaluation of OED. To test the hypothesis that the progression model would differ in OED in the nonsmokers and those of smokers, they examined the chromosomal regions of tumor suppressor genes at 3p, 4q, 8p, 9p, 11q, 13q and 17p for LOH. All these variables were compared between OED in smokers and nonsmokers.[39]

The outcome of this study defined the clinicopathological features and the genetic profile of OED in nonsmokers and these findings correlated with the outcome, i.e., OSCC in a substantial number of patients in the longitudinal follow-up. The results of the findings in this study support that OED is seen in both smokers and in nonsmokers, but its occurrence in nonsmokers is associated with a higher risk of progression to OSCC, and they also progress quickly to cancer than in smokers. Although difference in the prediction models could not be ascertained, the authors interpreted that genetic alterations are similar between smokers and nonsmokers, regardless of how the changes have been acquired, i.e., which is considered to be mainly through environmental carcinogens, genetic predisposition or replicative errors. On the other hand, OED in nonsmokers may involve unique genetic mutations, which are driving the progression. These findings support the fact that the molecular pathogenesis between smokers and nonsmokers are indeed different.[39]

There is a shift in the paradigm of oral cancer management from surgery and radiation.[24] Precision medicine is evolving at preventive and management level. In principle, it considers genetic, proteomic, transcriptomic and metabolomic variability as well as environment and lifestyle influences that are unique to each affected individual.[40] Risk factors including habit history have to be evaluated for each and every individual. Precision medicine or personalized medicine also refers to the ability and design of the treatment plan which are unique and specific to the affected individual based on their predicted risk of disease.

LOH has been implemented as a strategic molecular biomarker to stratify the risk of the oral premalignant lesions in a clinical trial using epidermal growth factor receptor inhibitor, erlotinib.[36] In this study, the LOH-positive profile cases were confirmed to have LOH at 3p14 and/or 9p21 in participants with a given history of oral cancer, and in patients without a history of oral cancer, LOH profile was 3p14 and/or 9p21 with an additional chromosomal site, namely, 17p, 8p, 11p, 4q or 13q. The study showed a significant difference in the 3-year survival rate between the LOH-positive and LOH-negative groups. The 3-year cancer-free survival rate was significantly lower for LOH-positive compared with LOH-negative groups (74% vs. 87%, hazard ratio: 2.19; 95% CI: 1.25–3.83; $P = 0.01$). The authors have stressed that LOH testing in the management of oral premalignant lesions could be incorporated as a prognostic indicator in clinical practice. Cromwell *et al*. from British Columbia used a decision-analytic Markov model to estimate the cost-effectiveness of risk-stratified care using a LOH

genomic assay. In the experimental arm, patients with low-grade dysplasias were managed according to their risk profile using the assay.[41] Low- and intermediate risk patients were given longer screening intervals and high risk patients were treated immediately by surgery. Patients in the control arm had standard care with biannual follow-up appointments. Their study concluded that the use of LOH genomic assay in the management of low-grade dysplasia was associated with improved patient outcome and was economically viable.

The use of such an assay in future could provide "precision medicine," allowing for a change in follow-up frequency or early intervention as compared with current standard care. They also proposed that with further validation, LOH genomic assay could feature in the evolving model of providing care to patients with oral premalignant lesions.

Though LOH can be considered as a predictable biomarker, there are practical issues in extrapolating it in specific situations. As a biomarker, predicting MT, across the risk category of OED, the LOH profile, should be unique and reasonably consistent as dysplasia progresses from low to intermediate to high risk. The obtained information could be translated to clinical assessment, and thus, this biological knowledge could have therapeutic implications. In those cases, where the LOH profiles do not change with a clinically visible progression of the lesion which is consistent with the progressive histological changes, then there exists a subgroup of lesions which progress independent of their current identified model. The progression in this subset of cases thus could be by an alternative process.[28]

Furthermore, LOH profile of the primary lesion should be consistently present in the recurrent lesions and should be validated in the context of field cancerization.[28]

Another challenge is that the molecular signature of all known foci of LOH need not necessarily be associated with a precise defined rate of progression, since there is a probability that specific losses could be integral to MT.[28]

To conclude, LOH could be considered as a biomarker of MT in OED, provided that the limitations of its biological validity are addressed and clarified in future studies.

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Conflicts of interest

There are no conflicts of interest.

Umadevi Krishna Mohan Rao, Rooban Thavarajah, Elizabeth Joshua, Kannan Ranganathan

Department of Oral and Maxillofacial Pathology and Microbiology, Ragas Dental College and Hospitals, Affiliated to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, Tamil Nadu, India.
E-mail: umauvk@gmail.com

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