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The genetic basis of oral leukoplakia and its key role in understanding oral carcinogenesis

Short running title: Oral leukoplakia genetics

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Abstract
Oral leukoplakia (OL) is the most common oral potentially malignant disorder, with a global prevalence of 2-3%, variable malignant transformation rate and incompletely understood aetiology. Considering the subjectivity in oral dysplasia grading, other evaluation methods have been tested as predictors of malignant transformation. DNA ploidy status and loss of heterozygosity signatures have been shown to be good predictive markers of malignant transformation. However, effective markers to predict which lesions will progress to invasive carcinoma and by which mechanisms...
remains unclear. Recent evidence suggests that dysplasia progression to carcinoma occur through neutral clonal evolution (i.e., randomly). We focus on the genetic basis of OL, encompassing the gross chromosomal alterations and single-gene mutations, and discuss such alterations in the context of aetiology, clinical presentation, and progression. The deeper we understand the genetic basis of OL, the more we approach a better comprehension of the complex and poorly understood process of oral carcinogenesis.

**Keywords:** oral potentially malignant disorder; oral dysplasia; DNA ploidy; copy number alteration; loss of heterozygosity (LOH)

**Introduction**

Oral leukoplakia (OL) is the most common oral potentially malignant disorder (OPMD), with a prevalence of 2-3% and a malignant transformation rate of 3.5%.\(^1\) OL is a clinical term used to describe “white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”.\(^2\) Despite having no specific histological features, biopsy is mandatory for the definitive diagnosis of OL.\(^2\)

The OL aetiology is not fully understood, and while several cases are associated with tobacco use, others have no apparent cause.\(^2\) OL shows variable clinical appearance, varying from homogeneous to non-homogeneous.\(^2\) Proliferative verrucous leukoplakia (PVL) is a distinct and aggressive presentation of OL, more frequently occurring in elderly women. PVL develops at multiple sites, shows a high recurrence rate, and a strong propensity for malignant transformation.\(^3,4\)

OL shows epithelial hyperkeratosis with or without epithelial dysplasia.\(^2\) Approximately 50% of OL show epithelial dysplasia, which is graded as mild, moderate, or severe based on architectural and cytological alterations. Dysplasia is associated with an increased progression risk to oral squamous cell carcinomas (OSCC).\(^5\) Additionally, clinical features such as advanced age, female gender, non-smokers, size >200mm\(^2\), non-homogeneous appearance, and location on the ventrolateral tongue, floor of the mouth, retromolar and soft palate (i.e., high-risk sites) contribute to increasing the malignant transformation potential of OL.\(^1,6\)
Guidelines for the treatment of OL have not been established. Surgical intervention with surveillance is the treatment of choice, even though it is not able to prevent recurrences or the appearance of new lesions.

Considering the challenges in the diagnosis and prediction of malignant transformation of OL, understanding its genetic basis is crucial. OL lesions harbouring genetic alterations are considered cancerization fields with evidence of morphological changes\(^7\) (Figure 1A). Notably, OL may be surrounded by oral mucosa that also carry genetic alterations but are clinically/microscopically normal.\(^7\) The presence of a field of cancer-associated genetic changes, clinically visible or not, represents a risk for cancer development, but an in-depth discussion about the cancerization field is beyond the objectives of this review.

This review focuses on OL genetic basis, encompassing alterations from gross chromosomal alterations to single-gene mutations (Figure 1B). This theme has been poorly addressed by previous studies, and also, there are several complexity layers that surround the study of OL. Therefore, we also discuss these genetic alterations in the context of clinical presentation, progression or aetiology, in addition to addressing future perspectives in studies in this field. In the search strategy, the terms “genetics”, “genetic alterations”, “DNA ploidy”, “copy number alterations”, “loss of heterozygosity”, “exome” and “mutations” were used along with “oral potentially malignant disorder” OR “oral premalignant lesions” OR “oral precancer” OR “oral leukoplakia” OR “oral dysplasia”. Important references and information derived from background knowledge have also been included. There is variability in the literature around the types of lesions associated with the use of the term OPMD, which results in difficulties in limiting to only OL. Therefore, eventually the results could not be specifically discussed exclusively for OL in the present review.

DNA ploidy

DNA aneuploid cells have an abnormal number of chromosomes, arising from chromosomal missegregation. DNA ploidy abnormalities have been extensively studied as predictors of OL malignant transformation risk,\(^8\) using either image or flow cytometry, with good agreement in the results.\(^5,9-14\) However, in solid tumours, image cytometry tends to be more accurate in detecting multiple aneuploid peaks than flow
cytometry. Image-DNA ploidy has been reported as a reliable method to identify oral epithelial dysplasia with a high-risk of malignant progression.\textsuperscript{5,9-13}

In 2013, Sperandio and colleagues established the value of DNA ploidy analysis and dysplasia grading in a population with an overall low incidence and risk of development of OSCC.\textsuperscript{5} Latter, they focused on oral lesions considered to be at high-risk based on clinical information and biopsy results.\textsuperscript{13} Overall, they showed that DNA ploidy is a good predictor of malignant transformation. Additionally, they provide evidence that combining DNA ploidy analysis with dysplasia grading improves the predictive value when compared to each technique alone.\textsuperscript{5}

While a positive correlation between ploidy status and histological grading of OL/OPMDs was reported in studies using flow cytometry,\textsuperscript{14-17} this was not confirmed using image cytometry.\textsuperscript{9,10,16} A meta-analysis pooled five studies\textsuperscript{5,9-12} and concluded that aneuploidy is associated with a 3.12-fold increased risk of malignant progression, with higher probability of no occurrence of malignant transformation in diploid OPMDs than in aneuploid cases.\textsuperscript{8} However, diploid cases should be interpreted with caution because they occasionally suffer transformation.\textsuperscript{8}

Notably, DNA aneuploid OLs/OPMDs were more frequently observed at high-risk locations, such as the tongue and floor of the mouth, than in the palate, gingiva and buccal mucosa.\textsuperscript{15,16,18-20} Aneuploidy was also more frequently detected in non-homogeneous OL than in homogeneous ones.\textsuperscript{15} In non-smokers, DNA aneuploidy is mainly related to aging, with DNA diploid status being considered as low-risk for malignant transformation.\textsuperscript{17} These results support that DNA aneuploidy and risk to progression vary according to clinical features and habits.

DNA ploidy has also been evaluated by image cytometry in PVL.\textsuperscript{3,4} DNA ploidy was assessed in several samples of six PVL cases, with abnormal ploidy detected before malignant transformation in four cases, and a borderline result found in one.\textsuperscript{3} The authors concluded that both conventional histopathology and DNA ploidy proved useful in predicting the site of transformation.\textsuperscript{3} DNA ploidy was evaluated in samples of 21 PVL patients, with aneuploidy in 89% of the specimens.\textsuperscript{4} Aneuploidy was able to predict malignant transformation in 5/9 OSCC cases (55%).\textsuperscript{4} Although these findings reinforce the aggressive nature of PVL, according to a more recent systematic review, there is not enough evidence to correlate DNA ploidy status with its malignant transformation.\textsuperscript{21}

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A multiplex FISH panel has been recently used to detect aneuploidy at a single cell level in epithelial dysplasia, providing information on the spatial architecture of dysplasia not observed by other methods. Interestingly, aneuploid cells formed a minority cell population in all cases and were shown to be interspersed with normal diploid cells in the oral dysplastic epithelium. Notably, the authors also observed agreement in aneuploidy results by the FISH panel and image-based cytometry in 17 of 19 samples.

Telomere alterations

Telomeres are nucleoprotein complexes located at the ends of chromosomes. Loss of telomeric repeat sequences may lead to chromosome fusion and consequent chromosome instability, contributing to carcinogenesis. The impact of telomere length on the progression of OL has been poorly explored, and shortened telomere length in OL and adjacent mucosa compared to normal mucosa has been reported.

Copy number alterations

The genome of normal cells exhibits germline copy number variants (CNV), which are a source of genetic variation, and their counterpart in somatic cells is named copy number alterations (CNA). These genomic imbalances originate from gains, amplifications, deletions, and insertions of DNA sequences, which have the potential to promote gain or loss of function in genes related to proliferation, apoptosis, and cell cycle control.

EGFR copy number gain has been reported as a driver alteration in HNSCC, and therefore has also been investigated in OPMDs. EGFR copy number gain has been demonstrated in oral epithelial dysplasia and non-dysplastic homogeneous OLs. Such gains are more frequently detected in non-homogeneous than in homogeneous OL. Furthermore, OPMDs with increased EGFR copy number gain presented an increased malignant transformation risk. EGFR copy number gain has potential as a marker of OL malignant transformation, but additional studies including chemoprevention trials results are necessary.

Genomic imbalances of CDKN2A (9p21.3), RASSF1 (3p21.31), FHIT (3p14.2) and RB1 (13q14.2), have been reported in dysplastic OL that progressed to OSCC and in non-progressive dysplasia cases. While in dysplasia with malignant
progression CNA often involved multiple loci, in non-progressive dysplasia alterations tended to involve a single locus.\textsuperscript{30}

An analysis of the whole genome of 62 OPMDs with a more than 10 years longitudinal follow-up was carried out by tiling-path array comparative genomic hybridization.\textsuperscript{31} Low-grade dysplasia progressing to OSCC showed a higher abundance of genomic alterations when compared with non-progressive low-grade dysplasia,\textsuperscript{31} suggesting that CNA analysis in OL with low-grade dysplasia can be used as a predictive tool for malignant transformation.

An exome-wide study showed frequent DNA amplification of 7p11.2 (\textit{EGFR}) and 11q13 (\textit{CCND1}) in high-risk OPMDs\textsuperscript{32} in contrast to low-grade non-progressing lesions. Increased \textit{CCND1} dosage was associated with an 8-fold elevated risk of malignant progression and trisomy/polysomy of chromosomes 7 and 11 were more frequently observed in progressing lesions than in non-progressing and normal mucosa samples.\textsuperscript{29}

Array comparative genomic hybridization (aCGH) was used to assess CNA in 20 sequential progressive OLs and five same-site OSCC from five patients.\textsuperscript{33} Recurrent DNA copy number gains of 1p, 11q, 9q, 21q, and DNA losses at 5q and 16p were observed. Given that these CNA were identified in low to high-grade dysplasia samples and in their corresponding OSCC in 70\% of cases, a role in early proliferative process events with potential involvement in disease progression was suggested.\textsuperscript{33}

The genomic profiles of two OSCC, one co-occurring with an OL and the second being followed by an erythroleukoplakia after the primary tumour treatment were evaluated by aCGH.\textsuperscript{34} Both the OL and erythroleukoplakia cases showed more genomic imbalances than the respective invasive tumours.\textsuperscript{34} However, the limited number of samples evaluated does not allow any conclusions.

Whole exome sequencing (WES) was used to evaluate CNA in oral epithelial dysplasia and OSCC from 69 patients.\textsuperscript{35} Dysplasia samples were stratified in low- and high-grade. An overall increase in CNA was detected from low to high-grade dysplasia and OSCC. Only a minority of low-grade dysplasia samples showed CNA, while CNA profiling was very similar in high-grade dysplasia and OSCC.\textsuperscript{35}

Dual-colour FISH was used to assess the numerical and spatial patterns of CNA, revealing wide variation in copy number at different loci in oral epithelial dysplasia.\textsuperscript{22} Multiple copy number gain involved only \textit{CCND1} and \textit{EGFR}, with
patterns suggestive of a clonal architecture. While CCND1 amplification co-localized with severe dysplasia, EGFR amplification did not correlate with dysplasia.\textsuperscript{22}

Collectively, the studies that assessed CNA in OPMD, OL, and oral dysplasia showed gains and/or losses where oncogenes or tumour suppressor genes related to the carcinogenesis process are mapped. These alterations are more evident in severe dysplastic and/or progressive lesions,\textsuperscript{28,29,30,32} in those from high-risk sites,\textsuperscript{20} and in non-homogeneous lesions.\textsuperscript{27} However, CNA were also detected in non-dysplastic lesions,\textsuperscript{26,31} including cases that progressed to cancer.\textsuperscript{31} Overall, these studies showed an association between CNA and increased risk of progression, but longitudinal studies are necessary to validate these findings.

**Loss of heterozygosity studies**

Loss of heterozygosity (LOH) is a mechanism of inactivation of tumour suppressor genes during oncogenesis, whereby a locus that is heterozygous loses the wild type allele. Several studies have investigated LOH in OL/oral epithelial dysplasia.\textsuperscript{6,36-44}

In 1996, LOH was reported in OPMD for the first time, and a preliminary model of molecular progression from OL to OSCC based on LOH was proposed.\textsuperscript{36,37} The accumulation of these genetic changes was suggested to be associated with the risk of cancer progression, following a sequence in which mild dysplasia will progress to moderate and to severe dysplasia and OSCC. Currently, little evidence supports this hypothesis. Subsequent studies investigated the predictive value of LOH in the malignant transformation of OPMDs.\textsuperscript{38,40}

A dramatic difference between the LOH patterns of progressing and non-progressing OPMDs was reported. Cases with LOH at 3p and/or 9p showed a 3.8-fold increase in the relative risk of developing OSCC, and additional losses of 4q, 8p, 11q or 17p leading to a 33-fold increase in relative cancer risk.\textsuperscript{38} The prediction risk was further improved in a longitudinal study and validated in a retrospective cohort.\textsuperscript{40}

These studies showed that retention of 3p and 9p characterizes the low transformation-risk group; LOH on 9p or 9p with either LOH on 17p or 4q denotes intermediate-risk, and LOH detected in all three chromosome arms indicates high-risk OPMDs.\textsuperscript{40}

LOH at 4q, 9p, and 17p were equally predictive of progression from oral epithelial dysplasia to cancer in both, smokers and non-smokers.\textsuperscript{6} OPMD from
smokers and non-smokers showed loss of tumour suppressor genes loci, and cases that progressed either to high-grade dysplasia or to OSCC showed additional LOH. In agreement with that, a meta-analysis pointed to similar malignant transformation rates of OL from smokers and non-smokers, which might point to a genetic susceptibility.

Oral dysplasia from different anatomical sites may carry different progression risk, and higher LOH frequencies were observed in oral dysplasia from high-risk (floor of the mouth, ventrolateral tongue, and soft palate) compared to low-risk sites, mainly in mild and moderate dysplastic histological groups. However, there is scarce published information about the association between clinical presentation and the LOH profile of OL.

Previously, we investigated whether LOH profiles are associated with specific morphological features of dysplasia. We evaluated 29 microdissected OL samples using a panel of 11 microsatellite markers located on chromosomes 3, 9, 11, and 17. The presence of architectural and cellular changes, such as irregular epithelial stratification, drop-shaped rete ridges, and premature keratinization in single cells, showed an association with LOH at 3p14.2, 9p22, and 17p13.1, respectively. Therefore, these morphological features may harbour distinct molecular profiles, which add an extra layer of complexity in the interpretation of LOH data. Additionally, we showed the presence of inter- and intra-lesion molecular heterogeneity based on the LOH profile in OL samples. The heterogeneity should be considered in the development of strategies to target specific genes or pathways in the chemoprevention of malignant transformation.

In summary, LOH of markers mapped on 3p, 4q, 9p, and 17p, where tumour suppressor genes are mapped, has been implicated in the progression of oral epithelial dysplasia to OSCC. It has been shown that LOH profiles differ in high and low-risk sites and are associated with different histopathological parameters used to grade dysplasia. Furthermore, these profiles are similar in different grades of dysplasia and in OPMD from smokers and non-smokers.

**Gene mutations**

“The Cancer Genome Atlas” has provided insights into the genomic events associated with cancer and a similar initiative to catalogue genomic events in premalignant and early stage diseases has been proposed, the “Pre-Cancer

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Genome Atlas”. Although challenging, collaborative efforts may help to elucidate the molecular basis of OL and the malignant transformation process.

WES and whole-genome sequencing (WGS) were used in OSCC and adjacent dysplasia and TP53 was the unique gene frequently mutated at an early stage of OSCC development. A subclonal variation between dysplastic samples from the same lesion was observed, suggesting carcinomas often descended from the dysplasia subclone. Such finding reinforces that the relationship between dysplasia and carcinoma is complex, and not always a simple progression.

In a subsequent study, 16 oral dysplasia/carcinoma pairs were evaluated by WES and CNA was investigated in samples of oral epithelial dysplasia and OSCC from 69 patients (discussed in the “Copy Number Alterations”). TP53 mutations were shared by oral dysplasia and OSCC, suggesting their involvement in the early stages of cancer progression. However, pathway analysis revealed that OSCC developed differently in each patient. Also, all dysplasia samples contained somatic mutations that were absent in the related carcinoma. The number of shared mutations and the rate of accumulation of such mutations suggested that all samples contained a population of sub-clones that emerged without selective advantage. These findings suggest that the genomic alterations that drive OSCC occur in dysplasia and promote carcinogenesis by gradual random accumulation, consistent with the neutral tumour evolution. In line with that, in silico analyses of transcriptome data suggest that in some cases the premalignant lesions and the carcinomas are not clonally related to each other. Furthermore, the results of a recent study using a FISH panel in oral epithelial dysplasia reinforce that dysplasia progression to OSCC can occur through neutral clonal evolution (i.e., randomly), instead of through non-random selection. However, more studies are necessary to exclude the possibility of non-random selection of genetic alterations in OL progression. Neutral clonal evolution or non-random events may not be mutually exclusive, and they can occur simultaneously or at different stages during tumour evolution.

Recently, several sequential samples from 5 patients with OL that progressed and 8 patients with OL that did not progress to OSCC were evaluated by WES. A small number of genes involved in the progression status, including DNA damage repair genes, has been identified. Future studies enrolling more patients will help to strengthen such results.
Most of the molecular pathology studies have investigated only targeted genes in OL. TP53 mutations are one of the most critical genetic events in oral carcinogenesis and have been described in 37% of OSCC. Therefore, several studies have focused on detecting TP53 mutations in OPMDs.

TP53 mutations are a significant risk factor for OL malignant transformation, alone or in combination with LOH at 9p.41 Additionally, OL with or without dysplasia showed a similar mutational profile, with TP53 and KMT2C being the most frequently mutated genes.50

Collectively, the genetic studies in OL highlight the role of TP53 mutations in the early stages of carcinogenesis. Advances in molecular pathology studies in OL and further analysis using WES or WGS may uncover the molecular profile of OL that undergoes malignant transformation.

Concluding remarks

OL and OSCC can harbour genetic alterations common to both conditions. Although experimental data suggest that the malignant transformation of oral dysplasia to OSCC follows the neutral evolution model, more studies are necessary to exclude the possibility of non-random selection of genetic alterations in OL progression. Molecular predictive markers of malignant transformation of OL (DNA ploidy analysis and LOH signatures) have been established. However, at an individual level, no single marker can predict whether a given lesion will remain indolent or will progress to carcinoma. Collaborative initiatives devoted to sequence several cases of OL with and without dysplasia, with complete clinical information, and long-term follow-up could benefit patients with effective markers for diagnosis, prognosis, and therapeutic targets for prevention or treatment. The precise clinical characterization of the studied samples is of critical importance to translate molecular results into the clinics. In this scenario, the understanding of the complexity of genomic events that drive oral carcinogenesis may pave the way toward targeted molecular therapies.

References

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**Figure Legend**

**Figure.** 1. Oral leukoplakia (OL) main molecular alterations. (A) The field cancerization is represented in orange, and it is composed of cells carrying genetic alterations, either showing histopathological and clinical changes or not. Although genetic changes are usually present in OL, they can also happen in the clinically and histopathologically adjacent normal mucosa, taking part in the field cancerization. (B) DNA aneuploidy copy number alterations (CNA), loss of heterozygosity (LOH), and *TP53* mutations represent the core of genetic alterations of OL and oral potentially malignant disorders (OPMD). The presence of DNA aneuploid cells, with an abnormal number of chromosomes, is associated with an increased risk of malignant progression. The detection of CNA and the presence of LOH on chromosomes 3p, 4q, 9p, and 17p, where tumour suppressor genes are mapped, are associated with a higher risk of progression to OSCC. *TP53* is the most frequently mutated gene in oral dysplasia and OPMD, in agreement with the role of this gene in the oral carcinogenesis process.