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Mini Review

# Progression of Oral Leukoplakia to Cancer: Importance on Collection of Leukoplakia and Cancer Tissues from Disparate or Same Patients

Gourab Saha[1], Richa Singh[2], Shouvik Chakravarty[3,4] and Bidyut Roy[5*]

*[1]R and D Protein Biology, Thermo Fisher Scientific Inc., Bangalore, Karnataka, India; [2]Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY, USA; [3]Biotechnology Research Innovation Council, National Institute of Biomedical Genomics, Kalyani, West Bengal, India; [4]Biotechnology Research Innovation Council, Regional Centre for Biotechnology, Faridabad, Haryana, India; [5]Department of Physical Anthropology, Anthropological Survey of India, Salt Lake City, Kolkata, India*

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## Abstract

Oral squamous cell carcinoma (OSCC) is a predominant type of head and neck cancer in the Indian subcontinent, mostly observed among tobacco and/or alcohol users. Oral leukoplakia (OLK) does not seriously affect patients, so it is often ignored in treatment. Some studies have reported genomic alterations and expression deregulation that drive OLK towards OSCC, conducted in two types of studies based on sample collection from (a) disparate or (b) the same patients. Demographic, tobacco/alcohol habits and biological factors may vary significantly if OLK and OSCC samples are collected from disparate patients, but they remain consistent if both tissue samples are from the same patient. Earlier, both targeted candidate gene-based and large-scale omics-based studies identified somatic mutations in *TP53*, *CDKN2A*, and *PTEN*, as well as broad arm-level copy number alterations and epigenetically dysregulated genes in leukoplakia and tumor tissues from disparate patients. Recent omics-based studies have identified early *CASP8* somatic alterations, *APOBEC* mutagenesis, as well as dysregulated immune cell infiltration (decreased $CD8^+$ T cell abundance, enrichment of pro-inflammatory immune cells) as candidate driver events for oral tumor progression from leukoplakia in the same patient. Recent single-cell transcriptomic-driven studies have also identified immune-transcriptomic features as putative driving molecular events in oral tumor development and progression. Here, we reviewed reported differences in driving gene mutations and expression deregulations in disparate and same patient settings. We also highlighted the challenges in sample collection and the opportunity of genomics and transcriptome studies for their emerging role in early diagnosis and progression.

## Introduction

Studies relating to the progression of precancer to oral cancer were performed using two types of study designs: collection of precancer and oral cancer samples from the same or disparate patients. As a result, driver mutations as well as dysregulated expression in both types of tissues were observed to differ in these two patient settings. Most studies collected precancer and cancer tissues from disparate patients and reported somatic mutations in various genes such as *TP53*, *CDKN2A*, *PTEN*, etc., along with dysregulated gene expression.[1–4] However, recent studies have identified early *CASP8* somatic mutations as well as dysregulated immune cell infiltration as early driver events in the progression study from leukoplakia to oral squamous cell carcinoma (OSCC), collected from the same patients.[5] In addition to these differences, many other driving mutations and expression results were identified in the two types of patient settings.[6,7]

In this review, we will concentrate on genomic, transcriptomic, and epigenomic changes in the transformation of oral leukoplakia (OLK) to OSCC, reported in different studies either from the same or disparate patients. This review aimed to provide a concise overview of the molecular marker alteration events that drive precancerous tissues to become frank oral tumors.

**Keywords:** Oral leukoplakia; Oral squamous cell carcinoma (OSCC); Progression; Sample collection; Disparate patients; Same patients.

***Correspondence to:** Bidyut Roy, Department of Physical Anthropology, Anthropological Survey of India, EN-7-9, Sector –V, Salt Lake City, Kolkata 700091, India. ORCID: https://orcid.org/0000-0003-4117-5838. Tel: +91-9432076069, E-mail: bidyutroy8933@gmail.com

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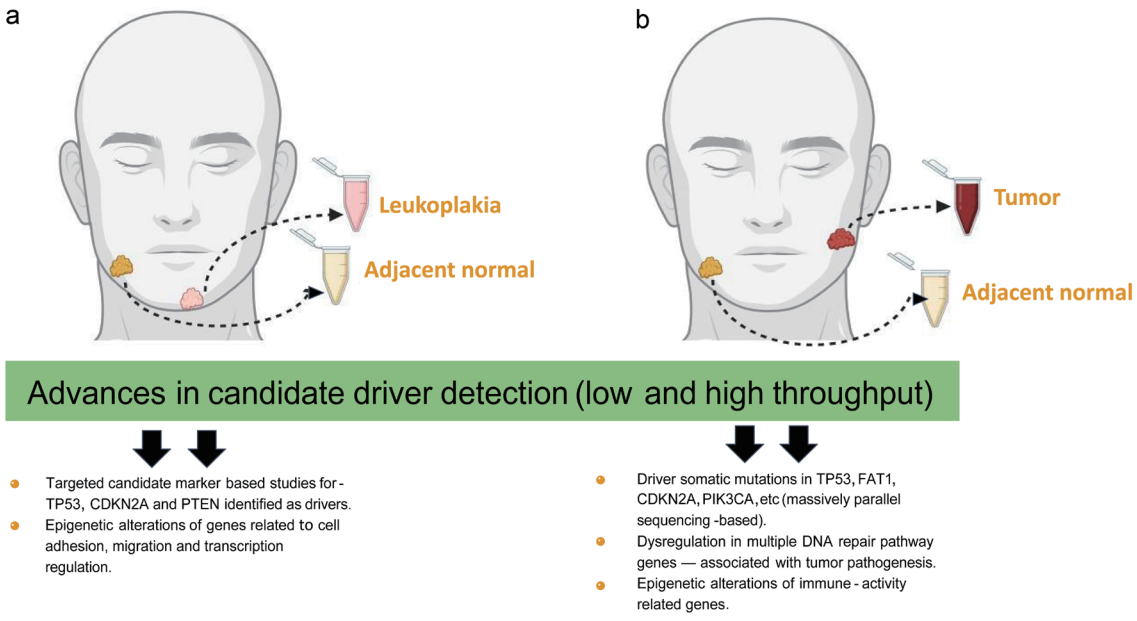


**Fig. 1. Candidate marker and omics-based findings associated with tumor progression in disparate patients with oral leukoplakia (a) and tumor (b) in their oral cavities.**

## OSCC

OSCC represents about 95% of head and neck cancers and is mostly observed in the oral cavity and oropharynx. It is one of the most prevalent cancers in Asia, with a global incidence of more than 350,000 new cases and 177,000 deaths every year.[8] Potentially malignant oral epithelial lesions (PMOELs) constitute a group of pathological conditions referred to as precancer. These include clinically observable oral mucosal lesions such as leukoplakia, erythroplakia, sub-mucosal fibrosis, and lichen planus, among others. Although the majority of PMOELs do not progress to cancer, a few have the potential to progress to oral cancer after several years if left untreated. It is now reported that up to 4–20% of OSCCs arise from preexisting PMOELs.[9]

## OLK

Histopathology of most leukoplakias typically shows benign keratosis, hyperkeratosis, or hyperplasia; only a minority of them exhibit some degree of dysplasia. As a general rule, the thicker the lesion, the greater the likelihood of finding dysplasia in the sample.

### *Tobacco use and OLK*

Tobacco use, whether smoked or in smokeless form, is a well-known associated risk factor for OLK. Additionally, the use of areca (betel) nut, as well as snuff and other forms of smokeless tobacco prevalent in many parts of the world, particularly in South and Southeast Asia, poses significant risks for OLK. In certain cultural practices in these regions, populations engage in reverse smoking by keeping the burning end of hand-made tobacco sticks (similar to small-sized cigarettes, locally known as *bidi*) within the mouth, leading to a wide range of oral mucosal lesions, including leukoplakia. In reverse-smoking populations, leukoplakias have a 19-fold increased risk of malignant transformation (MT) compared to those who use tobacco in other forms such as cigarette/*bidi* smoking or chewing. The overall MT rate varies across different studies, ranging from 0.13% to 34%.[10] The primary reason for this variability in MT is likely due to the diversity in tobacco/alcohol habits, environmental conditions, dietary habits, and other lifestyle factors.

## Study of genetic progression model of oral cancer

### *Collection of leukoplakia (OLK) and OSCC tissues from disparate patients*

More than 70% of Indian OSCC patients visit the clinics when the cancer has progressed to a late, aggressive stage, necessitating surgical intervention as the primary therapeutic option (Indian Council of Medical Research- buccal mucosa cancer registry data). Under the influence of multiple carcinogenic stimuli in the oral cavity, normal oral epithelium becomes dysplastic and may ultimately give rise to frank oral tumors. OLK patients are rarely followed up, resulting in the majority of omics-driven studies on oral cancer progression focusing predominantly on late-stage tumor samples. Most studies (largely candidate gene-based) aimed at investigating molecular genomic alterations in OLK and frank oral tumors have been conducted on samples collected from two groups of patients (i.e., disparate leukoplakia and cancer patients) (Fig. 1). Consequently, leukoplakia and cancer patients may exhibit different types and frequencies of tobacco and/or alcohol use, dietary habits, and environmental influences. Generally, multivariate analysis has been employed to normalize differences in habits and other factors observed between disparate OLK and cancer patients.

In an accepted model of progression from OLK to OSCC, specific genetic changes have been reported in stages ranging from mucosal epithelial cell hyperplasia to dysplasia, carcinoma *in situ*, and ultimately invasive carcinoma (Fig. 2).[11] Unlike other cancers, OSCC typically initiates with the inactivation of tumor suppressors such as *CDKN2A* and *TP53* in early stages, followed by *PTEN* in later stages. In addition to mutations, several loss of heterozygosity (LOHs) have been identified at each stage of malignant pro-

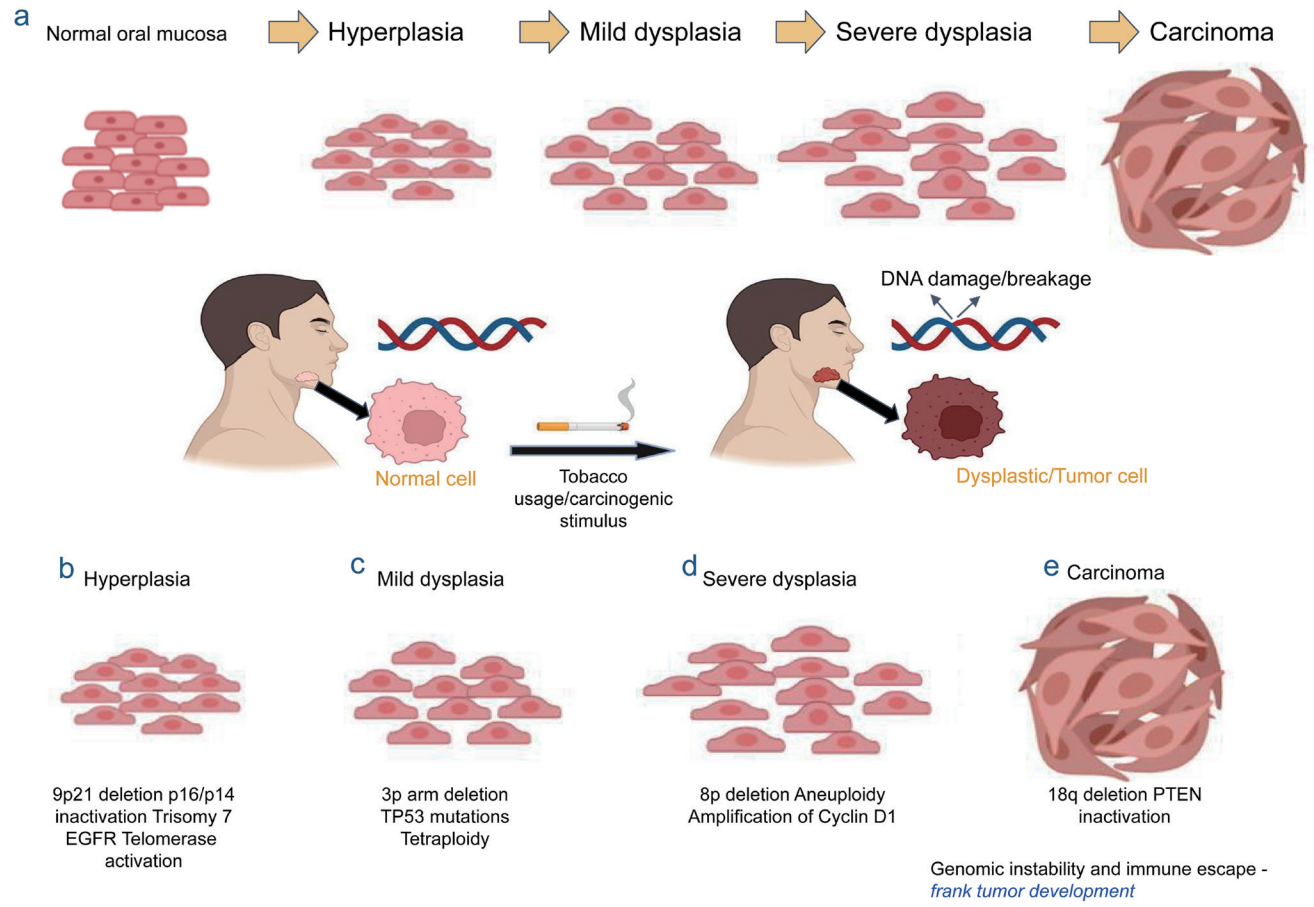


**Fig. 2. Oral cancer progression and associated genomic alterations.** (a) (top): Stepwise progression of oral squamous cell cancer, (below): Tobacco-induced carcinogens as a major factor behind dysplastic and cancerous transformation of oral epithelium; (b) Hyperplasia (9p21 deletion, p16/p14 inactivation etc.); (c) Mild dysplasia (3p arm deletion, TP53 mutation etc.), (d) Severe dysplasia (8p deletion, aneuploidy etc.); (e) Carcinoma (18q deletion, PTEN inactivation, genomic instability, immune escape etc.).

gression. Allelic losses at 3p, 9p, and 17p are frequently observed in dysplastic lesions, suggesting they could serve as early markers of carcinogenesis. Similarly, losses at 13q, 8p, and 18q are more common in carcinomas than in dysplasia, indicating their association with later stages of carcinogenesis. DNA aneuploidy has been shown to be a useful marker of MT from premalignant lesions to tumors; however, it has limitations in predicting cancer progression.[1,2] Additionally, LOH has been significantly associated with the progression of OLK.[12,13] Besides aneuploidy and copy number changes, single nucleotide polymorphisms (rs1473418 at *BCL2*, rs511044 at *CASP1*, and rs13010627 at *CASP10*) can modulate the risk of both OLK and OSCC in a large cohort of patients.[3] Genomic and transcriptomic profiles of gingivobuccal leukoplakia and OSCC patients from India have been studied,[4] identifying potential copy number alterations (CNAs) linked to disease progression. Their study on tissue samples showed strong correlations between expression and amplifications at 3q26.31 in both leukoplakia and OSCC. *MFAP5*, a secretory stromal protein overexpressed in leukoplakia and OSCC, may play a role in MT and could serve as a potential serum biomarker of cancer progression. However, the study also identified a few CNA regions that inversely correlated with gene expression, such as the down-regulation of *DERL3* located at 22q11.23.

Since 2010, unprecedented advancements in next-generation sequencing have transformed our understanding of cancer biology complexity, facilitating robust omics-based insights. Major national and international research consortia, such as the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA), have conducted large-scale multi-omics studies that help elucidate detailed mechanisms of cancer. Particularly in the Indian context, where gingivobuccal oral squamous cell carcinoma (OSCC-GB) poses a significant public health threat, studies within the ICGC-India consortium have comprehensively defined the somatic mutational landscape,[14] epigenomic perturbations,[15] and molecular genomic descriptors of lymph node metastasis primarily in late-stage Indian OSCC-GB patients.[16] The complex interplay among multiple components of the tumor microenvironment governs tumor progression. Many past studies have utilized a single diseased tissue biopsy per patient in their study design, potentially missing the complete omics spectrum of oral tumor progression from early dysplastic stages.

Epigenomic alterations may also drive tumor development and progression by regulating downstream gene expression programs. In an Indian cohort comprising 22 OLK and 74 OSCC-GB patients, several epigenetically altered genes (*FAT1, GLDC, HOXB13, CST7, CYB5A, MLLT11, GHR, LY75*) were identified, showing associations with expression dysregulation and patient survival.[17] In an effort to define the differentially methylated gene

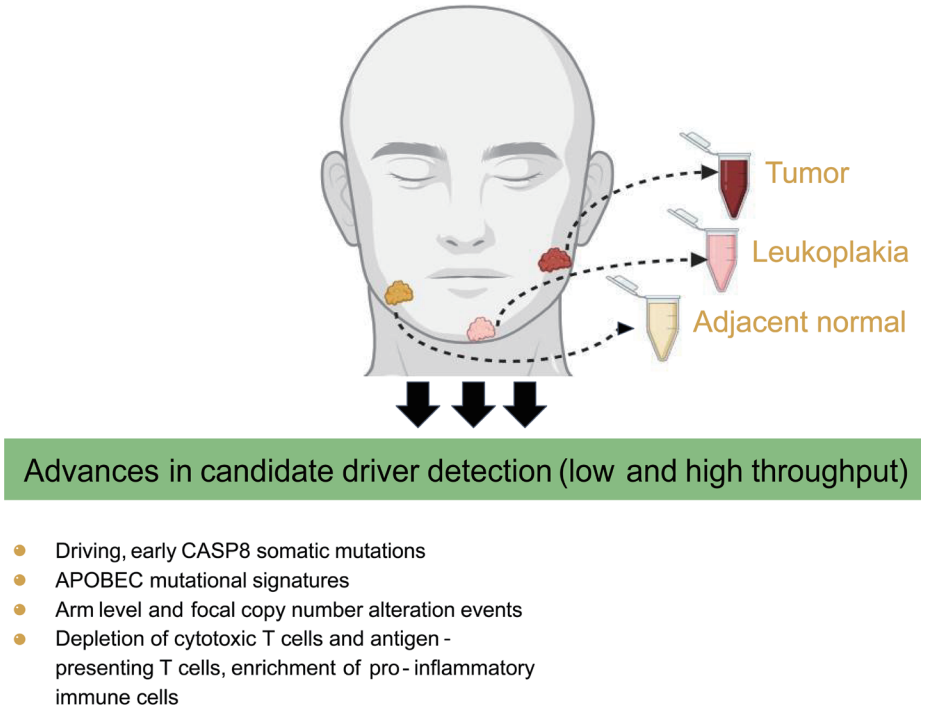


**Fig. 3. Molecular driving factors behind tumor progression in patients with presence of both leukoplakia and frank tumors in their oral cavity.**

landscape of proliferative verrucous leukoplakia, an aggressive subtype of OLK, investigators performed an integrated methylation and whole transcriptome study of 10 such cases, identifying expression dysregulation of genes associated with cell adhesion, migration, transcription regulation, and cellular signaling as features of oral precancer.[18]

### *Collection of leukoplakia (OLK) and OSCC tissues from the same patients*

Bulk transcriptome analyses often fail to characterize the individual gene expression programs active in dysplastic and tumor cells in fine detail. Recent technological advancements in the field of single-cell transcriptomics can help to address this question at a deeper level. Investigators analyzed single-cell RNA sequencing data from nine patients with both oral precancer and adjacent frank oral tumors and observed that a monocyte subtype associated with immune inhibition and vascular endothelial growth factor signaling modulation was a dominant feature of cancer initiation.[19] In an Indian cohort of oral cancer patients, including cases with or without concomitant precancerous oral submucous fibrosis lesions, several immuno-transcriptomic features such as enrichment of genes associated with partial epithelial-to-mesenchymal transition and varying levels of infiltration of cytotoxic T cells and macrophage polarization were identified as events associated with cancer initiation and progression.[20]

Sample collection of OLK and OSCC tissues for genomics and transcriptome studies from the same patients is rarely reported. Since patients with both OLK and cancer in the oral cavity are seldom available in hospitals, only a few could be obtained. However, when available, both OLK and OSCC tissue samples could be collected from these patients (Fig. 3) with proper ethical consent. In such studies, OLK and OSCC were exposed to identical doses and durations of tobacco and/or alcohol use and other environmental factors, as both affected tissues were collected from the oral cavity of the same patient. There are only a few studies aimed at identifying somatic mutations and alterations in expression in OLK and OSCC tissues collected from the same patients.

An interesting study performed a comparative genomic hybridization analysis of 25 sequential, progressive PMOEL and same-site OSCCs from five patients.[21] In 20 out of 25 sequential PMOEL cases, minimal recurrent DNA copy number gains were identified on the 1p region, with high-level amplifications observed at 1p35 and 1p36. Other DNA copy number gains were frequently observed at 11q13.4, 9q34.13, 21q22.3, 6p21, 6q25, 10q24, 19q13.2, 22q12, 5q31.2, 7p13, 10q24, and 14q22. In addition to gains, DNA losses were observed in more than 20% of samples on 5q31.2, 16p13.2, 9q33.1, 9q33.29, 17q11.2, 3p26.2, 18q21.1, 4q34.1, and 8p23.2. These CNAs were mapped across all grades of dysplasia that progressed to OSCCs, suggesting their potential association with OSCC progression. Furthermore, amplification of *BTBD7, KHDRBS1, PARP1*, and *RAB1A* genes was exclusively detected in both progressive leukoplakia and corresponding OSCCs, suggesting their possible association with disease progression.

Exome sequencing of non-dysplastic and dysplastic OLK reported that alterations in DNA damage repair (DDR) gene-related pathways highly impacted OLK progression to cancer compared to those that did not progress. Specifically, *BRCA1, BRCA2*, and other double-strand break repair Fanconi anemia (FA)/breast cancer (BRCA) pathway genes (including *FANCA*, *DCLRE1B*, *NEIL3*, *RPA4*, *MLH1*, and *CHEK2*) were identified as influential contributors in patients where OLK progressed to cancer development. Therefore, the molecular signature of a DDR-deficient profile could distinguish high-risk from low-risk OLK lesions. Additionally, this profile may positively influence management strategies, including surgical intervention, surveillance, and potential medical intervention with DDR pharmacotherapeutics. Drugs targeting

DNA damage pathways, such as *olaparib,* a Poly (ADP-ribose) polymerase (PARP) inhibitor, have demonstrated therapeutic benefits in BRCA-mutant ovarian, breast, and prostate cancer. In addition to the DDR gene profile distinguishing progressive OLK from non-progressive OLK, multivariate analysis was able to classify dysplastic OLK based on its progressive status using a signature of 15 genes (*FAAH*, *OR2A5*, *FAT1*, *DAGLB*, *PTPRZ1*, *OR2L2*, *ACOXL*, *C17orf78*, *CCDC149*, *DCLRE1B*, *FRMD4A*, *PRCP*, *DCST1*, *GPR128*, and *PIM3*). However, further investigation of this mutational signature of dysplastic OLK is warranted, as differences in gene expression profiles between dysplastic and non-dysplastic OLK were noted.

A group of 170 oral precancer samples (OPCs) from the original Erlotinib Prevention of Oral Cancer randomized clinical trial (hereinafter referred to as EPOC) study were analyzed for 201 cancer-related genes by next-generation sequencing.[22] Of these samples, a subset of 141 underwent RNA sequencing using the HTG EdgeSeq Oncology Biomarker Panel containing 2,560 transcripts. In their follow-up study, 73 OPC patients developed invasive cancer after a median of 7.3 years. From these patients, the evolutionary trajectory from precancer to cancer was profiled in 23 paired oral cancer samples. The results were compared with an independent set of 86 OPCs with RNAseq data and TCGA invasive oral cancer DNA/RNA dataset. The study reported C > T transition as the predominant substitution, similar to TCGA data. The top frequently mutated genes in OPCs were *TP53* (29%), *CDKN2A* (15%), *NOTCH1* (11%), and *PIK3CA* (7%), which were also frequently mutated in oral cancer from EPOC and TCGA studies. They observed a progressive increase in tumor mutation burden ($p < 0.05$) and frequency of high-risk TP53 mutations ($p = 0.02$) from hyperplasia to dysplasia and invasive oral cancer ($p < 0.05$). The median tumor mutation burden was higher (2.45 Mb) in OPCs that developed oral cancer compared to those that did not (1.22 Mb; $p < 0.01$). Patients with *TP53*-mutated OPCs had shorter oral cancer-free survival compared to *TP53* wild-type (Hazard Ratio: 1.81, 95% CI 1.13–2.90, $p = 0.01$). Similarly, another study investigated normal epithelium, dysplasia (premalignant lesion), and OSCC from the same surgical formalin-fixed paraffin embedded specimens from 19 human papilloma virus (HPV)-negative patients and reported differences in immune cell signatures among these three tissue types.[23] They also reported upregulation of *HOX* genes and downregulation of adherens junctions, suggesting potential treatment implications for oral dysplasia using these dysregulated genes.

A study from this laboratory examined somatic mutations using whole exome sequencing method from 27 patients carrying both OSCC and adjacent histopathologically confirmed OLK tissues.[6] Interestingly, *CASP8* was identified as the most frequently and early mutated gene in both OLK and OSCC tissues from the same patients. Almost 56% of tumor tissues (15/27) and 30% (8/27) of leukoplakia tissues had *CASP8* mutations. Importantly, all *CASP8* mutations (n = 8) in cancer and adjacent leukoplakia tissues had identical mutation sites and types, which were non-synonymous, frameshift, and stop gain mutations, suggesting functional damaging properties of these mutations. Moreover, mutated allele frequencies were significantly higher in most tumor tissues compared to adjacent leukoplakia tissues, suggesting that cells with *CASP8* mutations might gain a selective advantage during leukoplakia progression to the tumor.

Another group from the same geographical location in India performed whole exome sequencing of OSCC tumors, adjacent leukoplakia tissue, and blood DNA from the same patients.[7] They found that somatic mutations in protein-coding regions were more frequent in tumors (23/55) compared to adjacent leukoplakia tissues (12/37). They also reported a significantly higher ($p < 0.0004$) somatic mutation rate per Mb in tumors (0.14–8.84 Mb, mean = 2.88 Mb) compared to leukoplakia tissues (0.17–5.06 Mb, mean = 1.21 Mb). Interestingly, in an additional cohort (n = 11) of leukoplakia patients without tumors in the oral cavity, the mutation rate was even lower (0.03–0.89 Mb, mean = 0.39 Mb). These results suggest that the mutational processes driving MT might start at the leukoplakia stage, with mutations in genes such as *TP53, CASP8, FAT1, PIK3CA, NOTCH1, KMT2B, HRAS, ARID2, EPHA2, HLA-B*, and *TGFBR2* detected in both tumors and adjacent leukoplakia tissue. *FBXW7* mutations were exclusively detected in tumor samples. Consistent with a previous study,[6] they also observed frequent *CASP8* mutations in both leukoplakia and adjacent tumors. Interestingly, *TP53* mutations were more prevalent in OSCC tumors (39%) compared to adjacent leukoplakia tissues (14%), supporting the hypothesis of early somatic events in *CASP8* driving tumorigenesis. Collectively, these findings suggest that early mutations in selective genes in leukoplakia likely provide the initial impetus for malignancy. The mutational findings of *CASP8* in leukoplakia and tumor tissue were further elucidated in a subsequent transcriptome study in the same samples,[7] where genes associated with cell survival, migration, invasion, and cellular stemness were significantly upregulated in both tumor and leukoplakia tissues harboring *CASP8* mutations, but not in those without *CASP8* mutations. They also reported that 72% of oral cancer driver gene mutations were shared between tumors and adjacent leukoplakia located in close proximity in same patients. However, additional mutations in tumor tissues suggest that multiple driver mutations are required for eventual transformation into malignant tumors. Apart from single nucleotide variations, they observed that CNA events, including *EGFR* amplification and DNA repair gene deletions, were more frequent in tumors compared to closely adjacent leukoplakia tissues. It is noteworthy to mention that while we found *CASP8* mutations in 30% of leukoplakia and 56% of adjacent oral tumor tissues, *TP53* mutations were present in 14% of leukoplakia and 39% of adjacent oral tumor tissues,[7] despite the identical sample size in both the *CASP8* and *TP53* mutation studies. This suggests that *CASP8* mutation may be an early driver of oral cancer progression. Other investigators failed to detect this *CASP8* mutation in OLK, even though they used OLK and tumor samples from the same patients in their experiments. This discrepancy may stem from differences in study design, as we collected both leukoplakia and adjacent tumor tissues from the same patients at a primary hospital. However, whether the *CASP8* mutation is specific to the population remains unclear, as there are no reports of *CASP8* mutations in other Indian populations using the same study design. Collaboration studies have now been initiated with more samples from different hospitals using the same study design.

Functional *in vitro* studies have also implicated *CASP8* alterations in head and neck cancer progression. Inactivating *CASP8* makes HNSCC susceptible to necroptosis-mediated cell death.[24] *CASP8* mutations in the amino-terminal domain were shown to induce TRAIL (an apoptosis ligand)-mediated apoptosis in HeLa and HNSCC cell lines.[25] Overall, omics and functional assays provide evidence that early *CASP8* somatic alterations initiate oral tumor development and progression by regulating biological processes such as apoptosis, cellular migration, and invasion.

In conclusion, the aforementioned genomic, transcriptomic, and epigenomic events are likely essential for leukoplakia transformation into cancer. Importantly, based on mutational data, multiple research groups have estimated that it takes nearly three years with an 80% probability of progression from leukoplakia to malignan-

cy, suggesting a significant window for clinical observation and management, consistent with findings from previous studies.[26,27] Since leukoplakia patients are often followed up with minimal clinical intervention, understanding the multi-omic molecular genomic and transcriptomic landscapes in large cohorts is essential for defining and understanding marker sets that potentially regulate tumor progression.

## Conclusions

Currently, assessment and management of leukoplakia lesions are solely focused on histological findings. Despite knowing the poor prognosis of oral cancer, it is very important to introduce genomic tools in the management of leukoplakia in current clinical settings. Unfortunately, only a very few molecular genetic studies were performed on leukoplakia tissues, since leukoplakia, in its initial stages, does not bother the patients much. As a result, leukoplakia patients do not like to visit hospitals in this subcontinent. Thus, it was difficult to explore the actual scenario of progression because leukoplakia and OSCC tissue were collected from disparate patients in most of the previous studies. Patients were affected by diseases at different oral sites and had different doses and types of tobacco habits. However, it is now clear that *CASP8* mutation in leukoplakia plays an important role in progression, as the study was performed collecting both leukoplakia and OSCC from the same patients.

Although difficult to collect, recently researchers started to study the progression of leukoplakia to OSCC grown in the same patients where both the leukoplakia and OSCC tissues were induced by identical oral habits. Again, sample sizes and heterogeneity of the OLK and tumors are significant challenges, but these challenges might be overcome by collaborating with different hospitals and characterizing more samples using same multi-omics techniques. Few molecular signatures, such as *CASP8* mutation and associated increased migratory potential of oral epithelial cells, were identified in leukoplakia, and these signatures may be responsible for the progression of OLK to oral cancer. Screening of these markers will definitely help clinicians for patient stratification and therapeutic management. Thus, in the future, genomic studies on leukoplakia and their follow-up studies will uncover the exact molecular landscape of MT.

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## Conflict of interest

Gourab Saha is employed by Thermo Fisher Scientific Inc. The other authors declare no conflict of interest.

## Author contributions

Study concept and design (BR); acquisition of data (GS, SC); analysis and interpretation of data (GS, SC, BR); drafting of manuscript (GS, RS, BR); critical revision of the manuscript for important intellectual content (BR, SC); administrative, technical or material support (GS, SC, BR); study supervision (BR). All authors have made a significant contribution to this study and approved the final manuscript.

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