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Epigenetic Mechanisms of Oral Leukoplakia: A Systematic Review

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ABSTRACT

Objectives: Oral leukoplakia (OL) is the most common type of oral potentially malignant disorder and has a malignancy transformation rate ranging from 0.1% to 17.5%. The rates of malignant transformation of OL to oral carcinoma can reach as high as 70%. Epithelial dysplasia in OL is linked to progression toward malignant transformation. We performed a systematic review (SR) to identify the epigenetic alterations associated with OL and their role in its progression.

Methods: The study protocol was registered in PROSPERO, ID: CRD42024497161. We searched PubMed, MEDLINE, Scopus, ScienceDirect, Web of Science, ClinicalKey and Wiley databases from 2018 to 2023. This SR was conducted following PRISMA guidelines, and articles were selected based on predefined inclusion and exclusion criteria.

Results: Seven studies met the inclusion criteria. Results documented that epigenetic mechanisms underlying malignant transformation of OL involved hyper and hypo methylation of CpG promoter in tumor suppressor gene, enhancer of zeste homolog 2 (EZH2) expressing cells in both the epithelium and connective tissues, methylation in retinoic acid receptor-beta (RARβ) promoter, involvement of non-coding miRNA which correlated positively with the degree of dysplasia, while salivary miRNA demonstrated potential as biomarker for the diagnosis and follow-up of OL patients.

Conclusion: Epigenetic non-coding miRNA biomarkers have potential mechanistic role in the early diagnosis of OL and serve as prognostic markers for its malignant transformation.

1. INTRODUCTION

Oral leukoplakia (OL) is the most common potential premalignancy in the oral cavity. OL is considered an intermediate stage. The cause of OL varies, and can be attributed to multiple factors, with many instances

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being of unknown origin. An annual incidence rate of malignant transformation from OL ranges between 6.2 and 29.1 cases per 100,000 people. Therefore, a sensitive and reliable method is greatly needed for the early detection of progressive malignant transformation of dysplastic OL (Downer et al., 2005; González-Arriagada et al., 2024; Malouf et al., 2013).

The use of tobacco, whether in smoked or smokeless forms, along with the consumption of areca (betel) nut preparations and alcohol, poses a significant risk and has a direct impact on the dynamic regulation of gene expression through epigenetic mechanisms. Additionally, the use of snuff and other types of smokeless tobacco further contributes to this concern. The modifications involved in epigenetics include DNA and RNA methylation, histone modifications, and the effects of non-coding microRNA (miRNA). Together, these factors regulate the expression and repression of genes within the genome. Abnormal changes in epigenetics are dynamic and can be inherited, leading to inappropriate gene expression that may promote tumorigenesis. However, epigenetic modifications are reversible and can be treated through pharmacological and dietary interventions (Kanherkar et al., 2014; Gasche et al., 2012).

The underlying molecular mechanisms responsible for the malignant transformation of OL is still not understood. Identifying epigenetic molecular markers associated with a higher risk of malignant transformation of dysplastic OL cells might be useful (Abe et al., 2016). Gaining deeper insights into these molecular events is crucial for understanding the early stages of oral cancer development. This knowledge holds promise for improved diagnostics, which can aid in early detection and risk stratification for therapeutic interventions (Kumari, et al., 2022).

Furthermore, it is of fundamental importance that early diagnosis is the most effective and economical method to reduce the incidence, mortality, and morbidity associated with oral cancers while also minimizing treatment-related complications. Thus, the present systematic review was conducted to study the molecular events of epigenetic effects during the malignant transformation of epithelial OL.

2. MATERIALS AND METHODS

The study protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO), ID: CRD42024497161. This research serves as the second part of our previous report on the genetics of OL (Kazi et al., 2024).

2.1 Search strategy

The articles were systematically searched in accordance with the PRISMA 2020 guidelines. It was conducted in three stages involving article i. identification, ii. screening, and iii. included, in parallel with the predefined inclusion criteria (Table 1). In the identification stage, articles were searched through PubMed, MEDLINE, Scopus, ScienceDirect, Web of Science, ClinicalKey and Wiley databases, from the year 2018 to 2023, based on Booleans ‘epigenetic’ AND ‘oral dysplastic lesions’ AND ‘oral leukoplakia’. The articles obtained from all databases were downloaded and transferred to the reference management software EndNote to ease the tracking of articles, in terms of the number of articles identified and the number of duplicates removed, as well as for the purpose of sharing the access of EndNote library between the reviewers at the end of article screening.

2.2 Procedure for study selection

Two independent reviewers were involved in the identification phase (article search through databases), and the same independent reviewers were involved in the screening phase (article with title, abstract and

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full text available, that comprises predefined inclusion criteria). Addition of one more independent reviewer (3 reviewers) was involved in the included phase (an article that concludes the predictive or diagnostic markers of OL). Discrepancies were resolved by the addition of one more independent reviewer (4 reviewers) through inter-examiner calibration. The search strategy was based on Boolean 'epigenetic' AND 'oral dysplastic lesions' AND 'oral leukoplakia'. The selection criteria include (i) Type of study, (ii) Type of report, (iii) Publication date and (iv) Language. The inclusion and exclusion criteria used to select studies are shown in Table 1.

Table 1. Inclusion and exclusion criteria.

	Inclusion criteria	Exclusion criteria
i. Type of study	a. Prospective studies that analyse progression of oral leukoplakia with long-term follow-up outcomes, or b. Retrospective studies comparing the groups with and without oral leukoplakia.	Single case studies.
ii. Type of report	Studies that report on: a. Predictive markers (for Prospective studies), or b. Diagnostic markers (for Retrospective studies).	Studies that do not report on signaling pathways/molecular markers.
iii. Publication Date	Articles between 2018 - 2023.	Articles published before 2018, and after 2023.
iv. Language	Articles in English language.	Articles in other languages.

2.3 Strategy for data synthesis

2.3.1 Details of demographic characteristics of the study patients

The details of demographic characteristics of the study patients involving age, gender, country, cigarette smoking, alcohol consumption, areca (betel) nut use, or mixed habit were recorded.

2.3.2 Determination of outcome determinant and histological features

The outcome parameters investigated in the selected studies were the epigenetic factors of signaling pathways as hallmarks of OL. The outcome determinants include upregulation or downregulation of the predictive or diagnostic markers, concerning the histopathological features. The epigenetic factors and the risk for OL conditions were summarized, based on the authors and their findings.

2.3.3 Epigenetic factors, and the risk for oral leukoplakia mechanisms of pathogenesis.

The groups of the mechanisms of pathogenesis for OL conditions obtained from patients of each study were described.

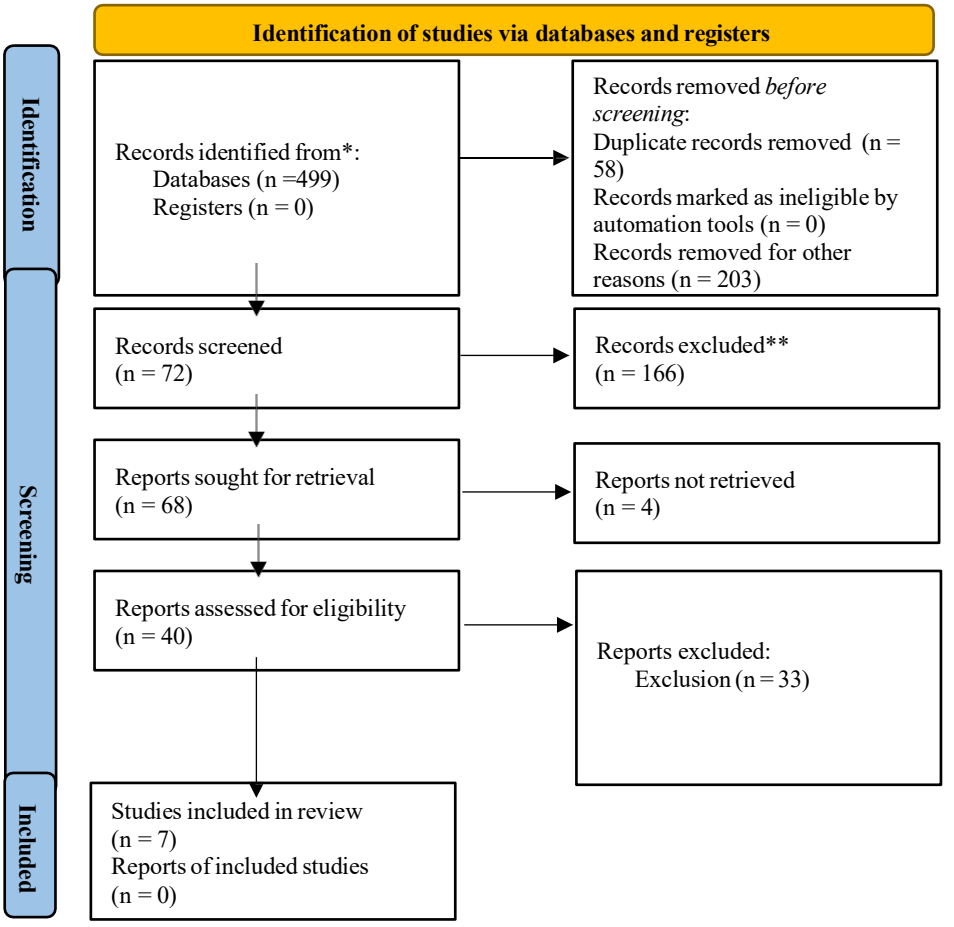
3. RESULTS

3.1 Articles selection

A total of 499 articles were retrieved during the Identification stage through database searches. After the Screening stage, duplicates were removed, and research articles published in English with full text available, reporting on prospective and/or retrospective studies involving humans, were selected, resulting

https://doi.org/10.24191/cos.v12i1.5653

in 68 articles. At the end of the third stage, inter-examiner calibration was done to finalize selected articles for the present review, where 7 research articles were finally selected for the present systematic review at the end of the included stage (Fig. 1).



*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).

**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.

Fig. 1. PRISMA 2020 flow diagram.

The search and selection of the articles were carried out in three stages. In the first stage (Identification), articles were searched through a database based on Booleans. In the second stage (Screening), the duplicates were removed, and research articles in the English language with full text available, which reported the findings on prospective and/or retrospective studies in humans were chosen. In the third stage (Included), only research articles that concluded on the predictive markers (for prospective studies), or diagnostic markers (for retrospective studies) were chosen. At the end of the third stage, inter-examiner calibration was done to finalize selected articles for the present review.

https://doi.org/10.24191/cos.v12i1.5653

3.2 Epigenetic factors, and the risk for oral leukoplakia conditions

The biomarkers of OL based on epigenetic factors, with and/or without dysplastic changes, are summarized in Table 2 and Fig. 2.

Table 2. Epigenetic factors, and the risk for oral leukoplakia conditions.

Epigenetic						Author
Biomarkers		Histological feature				
		Normal	Hyperplasia	Dysplasia	OSCC	
Hypermethylation	RARβ2			↓		Radhakrishnan et al. (2021)
	$P16^{INK4a}$			↑		Buenahora et al. (2021)
	FAT1		↑	↑		Inchanalkar et al. (2023)
	GLDC		↑	↑		
	HOXB13		↑			
	EZH2				↑↑	Ganesh et al. (2023)
Upregulation	miR-372	↓	↑↑			Tu et al. (2021)
				↑	↑	Tu et al. (2022)
	miR-10b	↓				Tu et al. (2021)
				↑	↑	Tu et al. (2022)
	circHLA-C	+	↑	↑↑		Xu et al. (2020)
Downregulation	miR-375	+	↓↓	↓	↓↓	Tu et al. (2022)

+ = expressed
↑ = increased
↑↑ = highly increased
↓ = decreased
↓↓ = highly decreased
- = no association

RARβ2: retinoic acid receptor-beta 2, p16INK4a: INK4 family member p16, FAT1: FAT atypical cadherin 1, GLDC: glycine decarboxylase, HOXB13: Homeobox B13, EZH2: enhancer of zeste homolog 2, miR-372: microRNA-372, miR-10b: microRNA-10b, circHLA-C: circular RNA HLA-C, miR-375: microRNA-375.

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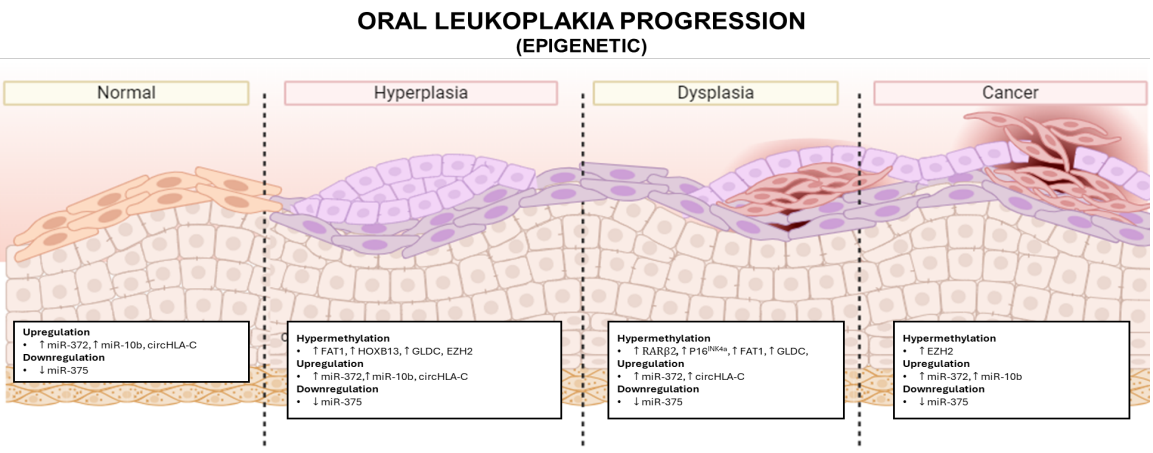

Fig. 2. Epigenetic factors and the risk for oral leukoplakia conditions.

miR-372: microRNA-372, miR-10b: microRNA-10b, circHLA-C: circular RNA HLA-C, miR-375: microRNA-375, FAT1: FAT atypical cadherin 1, HOXB13: Homeobox B13, GLDC: glycine decarboxylase, EZH2: enhancer of zeste homolog 2, RARβ2: retinoic acid receptor-beta 2, p16INK4a: INK4 family member p16.

3.3 Potential biomarker(s) of oral leukoplakia

The epigenetic mechanisms involved in the formation and progression of OL were DNA methylation, histone modification, and the non-coding RNAs regulation.

DNA methylation

Deoxyribonucleic acids (DNA) methylation adds chemical group to DNA, which turns off the gene. A study by Buenahora et al. (2021), investigated on the DNA methylation profile of OL samples according to expression of p16INK4a and HPV16 genotype (positive or negative). Methylation of p16INK4a loci is present in OL tissues, however the condition may not be associated with HPV infection. However, HPV infection in OL tissues induces modulation of genes related to immune system and regulation of cell cycle. Methylated loci of p16INK4a were also observed in HPV positive dysplastic OL samples, suggesting that a shift in methylation status may be important in OL progression to OSCC. In addition, hypermethylation of genes in HPV positive samples are associated with signaling by NOTCH1, Interferon-gamma and Interleukin-6.

Another study by Inchanalkar et al. (2023), investigated on the DNA methylation profile in HPV-negative OL with dysplasia and without dysplasia, and gingivobuccal cancer samples. Distinct methylation profile is present in both OL and gingivobuccal cancer, when compared to the normal oral tissue samples. Based on integrative analysis of genome, epigenome and transcriptome data, the study validated several candidate genes including FAT1, HOXB13, GLDC, CST7, CYB5A, MLLT11, GHR and LY75, of which their gene expression was found synergistically associated between copy number and DNA methylation changes. Further analysis demonstrated FAT1 hypomethylation, and HOXB13 and GLDC hypermethylation to be associated with carcinogenesis.

Another study by Radhakrishnan et al. (2021), who investigated on the promoter methylation status of the retinoic acid receptor-beta 2 (RARβ2), revealed that RARβ2 expression is present in normal samples as well as OL with dysplasia, however lower RARβ2 expression was reported in tissues with immortal cells. In addition, the RARβ2 expression showed a heterogenous pattern, of which the proportion of RARβ2 expression in cells were variable and not associated with the degree of epithelial dysplasia, putting it as one of the important factors contributing to the lack of consistent effect of retinoids in chemopreventive trials.

https://doi.org/10.24191/cos.v12i1.5653

Besides, another study by Ganesh et al. (2023) investigated on the enhancer of zeste homolog 2 (EZH2) gene, which is one of the epigenetic regulators that function to repress transcription. EZH2 gene provides instruction for making the enzyme histone methyltransferase, and play role in cell proliferation and modulation of immune response. EZH2, CD3, CD8, CD1a are expressed in non-dysplastic and dysplastic OL tissues. EZH2 is highly expressed in epithelial layers of OL tissue that had undergone transformation to OSCC, when compared to the samples that had not undergone transformation, however no difference was observed in the connective tissue layer. Positive correlations were obtained between the epithelial expression of EZH2 complex and with both CD3 and CD8. In addition, overexpression of EZH2 complex is associated with a 13-fold higher risk for developing OSCC.

Non-coding RNAs

Non-coding ribonucleic acids (RNAs) helps control gene expression by i. attaching to the coding RNAs, or ii. by recruiting other proteins to modify the histones, which turns off the gene. Tu et al. (2021) investigated the expression of a family of non-coding RNAs, the microRNAs (miRNAs). The study investigated the level of miR-375 in the saliva of OL patients with and without dysplasia, revealed a decreased level of salivary miR-375 in comparison to the matched control volunteers. In addition, a higher level of salivary miR-375 is present in the OL tissues without dysplasia, than the tissues with dysplasia, with lowest expression observed in patients who had undergone malignant transformation. This shows salivary miR-375 as a potential biomarker during long-term follow-up of OL.

An extended study also by Tu et al. (2022), showed that miR-10b, miR-372 and miR-375 are present in the cytobrushed samples taken from the normal part of mucosa of the OL and OSCC patients, while both miR-10b and miR-372 are upregulated in OL with dysplasia. However, only miR-10b is upregulated in OSCC, when compared to the control samples. In addition, in comparison to the controls, miR-375 was downregulated in both OL with dysplasia and OSCC samples. This shows the involvement of miR-10b in the early stage of oral carcinogenesis, and the important roles of miR-372 and miR-375 in the OL differentiation towards OSCC.

Besides, another study by Xu et al. (2020) investigated the expression of another family of non-coding RNAs, the circular RNAs (circRNAs). circRNAs helps to control gene expression through the formation of miRNA sponges. The expression of circRNA is present in the normal tissue samples, however a total of 366 circRNAs are significantly altered in the OL tissues with dysplasia, involving 65 upregulation and 301 downregulation of circRNAs transcripts. Further analysis demonstrates a strong association between the circular human leukocyte antigen-C (circHLA-C) with the degree of dysplasia. This study may thus provide evidence of circHLA-C as a potential diagnostic biomarker in OL.

The summary of the studies of epigenetics of OL are summarized in Table 3.

https://doi.org/10.24191/cos.v12i1.5653

Table 3. Summary of the studies of epigenetics of leukoplakia.

Author	Year	Study Design	Methodology	Study Interest	Results	Mechanism	Conclusion
Buenahora et al	2021	Cross sectional	Dysplastic tissues of OL. Immunohistochemistry. RRBS sequencing.	Development of oral dysplasia caused by human papillomavirus (HPV)	HPV infection in OL modulates immune system genes and cell cycle.	Hyper and hypo methylations of CpG promoter of tumor suppressor gene p16INK4a and histone modifications are associated with anti-viral gene programs.	Deactivation of tumor suppressor genes through global methylation in DNA and histone modification may lead to malignant alterations in cells. Unveiling these mechanisms may aid in the development of novel diagnostic and prognostic markers, and therapies for malignancy.
Inchanalkar et al	2023	Demographic and clinicopathological study.	i. Integrative analysis of genome, epigenome, transcriptome, and genome-wide differential methylation profiles associated with oral premalignant lesions and gingivobuccal complex cancers. ii. Association of these profiles with clinical outcomes in GBC-OSCC patient.	DNA methylation patterns across different stages of oral carcinogenesis.	Genome-wide DNA methylation signatures, gene expression, and copy number changes associated with different stages of oral carcinogenesis.	Both OL and gingivobuccal (GBC)-OSCC shared hypermethylated promoters involved in the regulation of transcription, gene expression, biosynthetic and metabolic processes. The hypomethylated promoters were associated with development, cell adhesion, and regulation of immune-mediated responses, contributing to carcinogenesis.	Methylation signature of OL and GBC-OSCC can be used for identifying high-risk precancerous lesions having the potential of malignant transformation. A total of 32 marker genes with potential prognostic value in GBC complex cancers were identified. Integration of data across the genomic, epigenomic, and transcriptomic levels may help to identify novel genes with prognostic and therapeutic potential in BGC cancer.

https://doi.org/10.24191/cos.v12i1.5653

Radhakrishnan et al	2021	Chemoprevention trial study.	Demethylating agents 5-AZA-CdR in combination with all- trans-retinoic acid (ATRA) induces re-expression of retinoic acid receptor-beta (RARβ), reverses immortalization, and reactivates senescence program in a panel of immortalised primary cultures.	To confirm the de novo methylation of RARβ in oral epithelial dysplasia and investigate its role in cellular immortalisation and abrogation of cellular senescence.	Treatment of immortal dysplastic cells with 5-AZA-CdR in combination with ATRA leads to re-expression of RARβ.	RARβ promoter methylation in oral epithelial dysplasia was identified as key mechanism behind chemopreventive trial failure. Loss of certain retinoid receptors has been observed in both cell/tissue types during the progression of potentially malignant oral lesions (PMOLs) to OSCC.	i. Lack of consistent effect of retinoids in PMOLs seen in chemoprevention failure. ii. Combination with ATRA leads to the re-expression of RARβ, reverses inactivated p16 and activated telomerase, which are associated with immortalisation in dysplastic keratinocytes. iii. In epigenetic oral cancer pathogenesis, the combination of drugs has shown potential chemoprevention. iv. The lack of comprehensive studies to address variability of response particularly regarding effects on the cell cycle and replicative potential may have contribute to chemopreventive trial failure.

https://doi.org/10.24191/cos.v12i1.5653

Ganesh et al	2023	Retrospective	9 OL cases (tissue samples) that had transformed into OSCC and 9 that had not transformed.	Association between enhancer of zeste homolog 2 (EZH2) Expression with immune activation and cancer transformation in OL.	High EZH2 expression in OL epithelium is associated with a 13-fold increased risk of transformation to OSCC. EZH2 is involved in the regulation of genes associated with cellular proliferation, differentiation, and apoptosis.	Interaction of EZH2-expressing keratinocytes with immune cells.	EZH2-expressing cells in both the epithelium and the connective tissues of OL patients serve as predictive markers for cancer transformation.
Tu et al	2021	Case-control	Quantitative RT-PCR analysis of miR-375 in the saliva of OL patients and healthy controls to explore the potential application of salivary miRNA as a biomarker for detecting and monitoring OL to OSCC transformation over time.	To explore the potential of deregulated miR- 375 levels in saliva to serve as biomarkers for the early detection and prognostic indicators in OL patients.	Potential use of salivary miR-375 as a biomarker for the detection and long-term follow-up of OL.	Salivary miR-375 levels were decreased markedly in OL patients compared to controls. OL patients with non-dysplasia showed a higher abundance of miR-375 in the saliva than dysplasia patients, suggesting that salivary miR-375 is a more sensitive marker for OL. Patients with malignant transformation during the follow-up period showed lower expression of saliva miR-375 than others. MiR-375 expression was markedly decreased following treatment with miR-375 inhibitor.	The strong correlation between salivary and plasma miRNAs, suggests that multiple salivary miRNAs may enhance diagnostic accuracy and follow-up monitoring of OL patients. Developing new, specific biomarkers is critical for improving the diagnosis and prognosis of such OPMDs. Saliva, being in constant contact with oral tissues and easily obtained through non- invasive methods, serve as an ideal specimen for identifying potential biomarkers.

https://doi.org/10.24191/cos.v12i1.5653

Tu et al	2022	Case-control	Quantitative analysis to identify epigenetic changes in non-coding RNA alteration with the greatest potential for malignant transformation.	Clinical implications of aberrant non-coding miRNA as a feasible system for OL classification and determining its malignant potential.	i. miR-372 showed high specificity (92.3%) to discriminate dysplastic and non-dysplastic OL. ii. miR-375 was unable to distinguish dysplasia from non-dysplasia. iii. miR-375 expression was able to discriminate OSCC from OL lesions. iv. miR-375 expression exhibited high diagnostic accuracy, with a specificity of 97.06%.	Compared to adjacent normal tissues, head and neck squamous cell carcinoma (HNSCC) tissues exhibit lower miR-375 expression. The sampling of normal counterparts in OL or OSCC patients is clinically unavailable. especially for screening purpose. miR-375 expression demonstrated a potential control counterpart.	Dysplasia is a strong indicator of malignant changes in OL. The results suggest that control counterpart tissues may be influenced by field cancerisation, which carries insidious abnormalities. This study also demonstrates that the combination of miR-375 and miR-372 enhances diagnostic accuracy.

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Summary of the mechanistic insights:

A. The deactivation of tumor suppressor genes through global DNA methylation and histone modification may facilitate the development of novel diagnostic and prognostic markers, and therapies for OL transformation into malignancy.

B. Integrating data across the genomic, epigenomic, and transcriptomic levels may help identify novel prognostic and therapeutic genes.

C. RARβ-promoter methylation in oral epithelial dysplasia identified as a key mechanism of chemopreventive trial failure. This highlights potential of combining drugs as chemoprevention.

D. EZH2-expressing cells in both the epithelium and connective tissues of OL patients serve as predictive marker for cancer transformation. It also indicates risk assessment markers for OL progression.

E. Dysplasia is a strong indicator of malignant transformation in OL. A control counterpart tissue might be influenced by field cancerisation, which carries insidious abnormalities.

F. Multiple salivary non-coding miRNAs might provide a better power of discrimination for the diagnosis of OL and follow-up of OL patients.

G. Mapping and constructing ceRNA which includes miRNA sponges or antagomirs (ncRNAs/miRNAs/mRNAs), may provide a promising approach for developing diagnostic and prognosis markers, as well as therapeutic targets.

(Buenahora et al., 2021; Inchanalkar et al., 2023; Ganesh et al., 2023; Radhakrishnan et al., 2021; Tu et al., 2021; Tu et al., 2022; Xu et al., 2020).

4. CONCLUSION

This systematic review showed that epigenetic alterations contributing to the malignant transformation of OL cells include hyper and hypo methylations CpG promoter of tumor suppressor gene, the expression of enhancer of zeste homolog 2 (EZH2) in both the epithelium and the connective tissues, methylation in the promoter region of retinoic acid receptor-beta (RARβ), the involvement non-coding miRNA that correlate positively with the degree of dysplasia, and salivary non-coding miRNA which demonstrate potential as biomarkers for the diagnosis and follow-up of OL patients. Furthermore, non-coding miRNA showed a feasible classification system for assessing the malignant transformation potential of OL.

Since epigenetic alteration is reversible, unlocking the mechanisms of epigenetic modifications in OL cells to malignancy is warranted. Mapping and constructing competitive endogenous RNA (ceRNA) systems for OL could offer promising targets for early intervention against malignant transformation (Tang et al., 2022; Vatsa et al., 2023).

In the future, advancements are expected to extend beyond the discovery of epigenetic biomarkers related to the malignant transformation of OL cells. Research will focus on exploring their potential mechanistic roles, which will aid in implementing this knowledge for early diagnosis, prevention, therapeutics, and prognosis. Additionally, there will be efforts to investigate their combination with chemotherapy (Lu et al., 2020).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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CONTRIBUTION OF AUTHORS

Jamil Ahsan Kazi. Conceptualization, performed the analysis, wrote, review and editing the manuscript. Corresponding author. **Noor Azliza Wani Abd. Aziz**. Designed the study, collected data, performed the analysis, wrote the manuscript. First author. **Nur Hayani Batrisya Mohd Rosli**. Collected data and performed the analysis. **Nur Sabrina Nazri**. Collected data and performed the analysis.

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5. APPENDIX

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