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Review Article

Genetics Section

Methylated DNA in Oral Brush and Salivary Rinse as a Non-invasive Biomarker in the Diagnosis and Prognosis of Oral Cancer: A Narrative Review

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ABSTRACT

Oral cancer is one of the significant health challenges globally, with late-stage diagnosis contributing substantially to poor prognosis and limited survival rates. Although tissue biopsy is the current gold standard for diagnosis, its invasive nature and limited sensitivity for early lesions restrict its effectiveness in routine screening and longitudinal monitoring. These limitations have driven growing interest in non-invasive molecular approaches that could support earlier detection and more effective clinical monitoring. Deoxyribonucleic Acid (DNA) methylation, a well-established epigenetic modification involved in gene regulation, has emerged as early and consistent events in oral carcinogenesis. Aberrant methylation of Tumour Suppressor Genes (TSG) DNA repairs genes, and other regulatory elements have been consistently reported in Oral Squamous Cell Carcinoma (OSCC). Importantly, these methylation changes can be detected not only in tumour tissue but also in non-invasive oral samples, such as oral brush biopsies and salivary or oral rinse specimens. This narrative review examines the diagnostic and prognostic potential of DNA methylation biomarkers detected through non-invasive sampling methods such as oral brush and salivary rinses. We summarise key findings from existing studies and discuss the relative strengths and limitations of these sampling approaches. Oral brush-based assays generally provide higher sensitivity due to enriched epithelial cell content and lesion-specific signals, whereas salivary and oral rinse-based assays offer greater feasibility for large-scale screening and repeated follow-up. Several multi-gene methylation panels have demonstrated encouraging performance in distinguishing malignant and potentially malignant lesions from normal oral mucosa, as well as in predicting disease recurrence and patient outcomes. Overall, methylation-based assays using oral brush and salivary rinse samples represent promising adjunctive tools for oral cancer detection and monitoring. While further validation, standardisation, and regulatory approval are required before routine clinical implementation, the integration of methylation-based assays into clinical practice holds the potential to significantly advance the early diagnosis and personalised management of oral cancer.

Keywords: Cancer prevalence, Deoxyribonucleic acid, Epigenetic alteration, Smoking

INTRODUCTION

Oral cancer remains among the most prevalent cancers affecting the global population, with an estimated incidence and mortality of 0.3 and 0.1 million, respectively [1]. Despite constant efforts to tackle the disease, most cases are frequently identified at late stages, and the five-year survival rate of the cancer has remained at 68% and below [2]. The risk factor for oral cancer varies from region to region. In Western countries, drinking and smoking habits have primarily been connected to oral cancer [3]. In Asian countries, tobacco and betel nut chewing, and smoking are the main causative factors [4]. Apart from these, *Human papillomavirus* (HPV) infection is also another factor contributing to the cancer, especially in developed countries [5].

During carcinogenesis, the very first and most common changes are epigenetic changes. DNA methylation alterations are the most common epigenetic alteration in cancer [6]. In oral cancer, extensive studies on genes altered by DNA methylation have been undertaken. Genes essential in mitigating tumourigenesis, like TSGs, cell cycle genes, and DNA repair genes, are reported to be aberrantly methylated in oral cancer [7].

Oral cancers are often preceded by morphological lesions known as Oral Potentially Malignant Lesions (OPML) which include Oral Leukoplakia (OL), erythroplakia, Oral Submucosal Fibrosis (OSMF), and lichen planus. A recent study involving 22 surveys reported the prevalence of OPML as 4.47% (95% CI=2.43-7.08)in which OSMF had the highest prevalence of 4.96% (95% CI=2.28-8.62) followed by OL with 4.11% (95% CI=1.98-6.97) [8]. Two recent systematic reviews showed that oral lichen planus had a malignant transformation rate of 1.09% [9] and 0.9% [10]. For OSMF, studies from different populations showed a malignant transformative rate ranging from 1.9% to 9.13% [11,12]. The malignant transformation rate of OL was found to vary from 0.13 to 34% according to a systematic study [13]. Erythroplakia malignant transformative rate was 2.6% to 65% with a mean of 30±0.2% [14]. The above variability makes it challenging to accurately predict which lesions will progress to cancer, often resulting in delayed diagnosis and poor prognosis.

DNA methylation represents an early epigenetic alteration in carcinogenesis and may arise before recognisable histopathological changes. Such methylation abnormalities have been reported in OPMLs, even in the absence of dysplasia, and may evolve dynamically during disease progression. Hypermethylation of TSGs such as p16 has been detected in OPMLs that later progressed to malignancy. Hall GL et al., reported p16 promoter hypermethylation in 57% of transforming Oral Epithelia Dysplasia (OED) versus 8% of non-transformers [15]. Cao J et al., observed p16 methylation in 41% of OED, with higher malignant progression in methylated lesions (43.8% vs 17.4%; adjusted OR=3.7; sensitivity 63.6%, specificity 67.9%) [16]. A multicentre cohort similarly showed increased transformation in p16-methylated OED (27.1% vs 8.1%; adjusted OR=4.6; sensitivity 62%, specificity 76%) [17]. These studies indicate that methylation in OED, especially p16 methylation

have shown a significantly higher risk of progression compared to methylation-negative lesions.

In diagnosing oral cancer, clinicians employ visual inspection of the oral cavity, biopsy sampling and histological evaluation [18]. Though the former is non-invasive, its specificity is questionable. It depends on the expertise and opinion of the examiner, while the latter, though invasive, is dependable and still the most accepted method for identifying oral cancer [19]. Oral cancer tissue and associated precancerous lesions have been the subject of numerous DNA methylation investigations. But not much progress has been made on the potential use of oral brush and salivary rinse to detect DNA methylation. These methods are non-invasive and can greatly improve the burden on the patient and remove the barrier of patient reluctance in diagnosis [20]. These methods will help in the early diagnosis of the cancer and help clinicians plan the treatment and prognosis accordingly, improving the overall cancer incidence scenario.

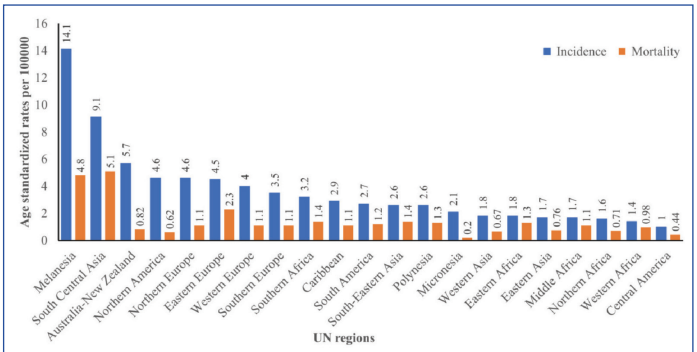
Given this context, the present narrative review's objective was to clarify the possible application of oral brushes, and salivary rinse as a non-invasive alternative in the diagnosis and prognosis of oral cancer using methylated DNA as a biomarker.

MATERIALS AND METHODS

A targeted literature search was conducted in PubMed, Scopus, Web of Science, and Google Scholar to identify studies investigating methylated DNA markers in oral brush and salivary rinse samples for oral cancer diagnosis and prognosis. Keywords included oral cancer, OSCC, DNA methylation, salivary rinse, oral brush, non-invasive biomarkers, diagnosis, and prognosis. Studies that were in English language and published between 2000 and 2025 were considered. Studies that examined DNA methylation in human salivary rinse or oral brush samples and reported diagnostic or prognostic relevance were considered. Studies based solely on tissue, blood, laboratory models, or lacking primary data were excluded. The eligible studies were screened and relevant information was extracted to synthesise current evidence. Across the included studies, comparison groups were not uniform and varied by study design. Controls commonly included healthy individuals, patients with benign oral lesions, and those with OPMLs or dysplastic lesions. Several studies also compared methylation patterns across normal mucosa, dysplasia, and OSCC to capture changes along disease progression.

Epidemiology of Oral Cancer

Globally, as per the International Agency for Research on Cancer-World Health Organization (IARC-WHO), 2022, oral cancer incidence is the highest in the Asian continent with South Central Asia occupying the topmost position within the region [1]. In terms of the Age Standardised Rate (ASR) of incidence, Melanesia had the highest Age-Adjusted Rate (AAR) of 14.1 followed by South Central Asia with 9.1 [Table/Fig-1]. Country-specific incidence is the highest in India, followed by China. The prevalence of the condition



[Table/Fig-1]: Age Standardised Rates (ASR) of incidence and mortality of oral cancer in UN regions (both sexes). The blue colour bars represent the incidence while the orange colour bars represent the mortality. Data source: Global Cancer Observatory; Cancer Today, 2022 (IARC-WHO) [1].

is most noted in individuals above 65 years of age and increases substantially with age [1]. However, among younger adults aged 44 years and below, a concerning rise in OSCC has also been reported [21]. Multiple factors have been investigated to explain this trend, including lifestyle-related exposures and viral infections such as high-risk HPV [22]. Although HPV-16 DNA has been detected in a minority of OSCC cases, current evidence indicates that its aetiological contribution is limited and heterogeneous, and HPV positivity frequently co-exists with established risk factors such as tobacco use and smoking-related behaviours rather than acting as an independent causal driver [5,23].

DNA Methylation and Cancer

The DNA methylation is one of the epigenetic modifications where a methyl group is added to the fifth carbon of the cytosine base in the DNA, resulting in 5-methylcytosine. DNA methylation usually occurs in Cytosine-phosphate-Guanine (CpG) regions known as CpG islands [24]. Most promoter regions of genes are rich in CpG islands, and hence, methylation in these promoter regions regulates transcription of the genes [25]. DNA methylation in the promoter region prevents transcription of the genes, while the absence of methylation promotes transcription and subsequent expression of genes. Therefore, DNA methylation plays a vital role in regulating gene expression. Apart from this, during development, DNA methylation helps in establishing the genomic imprinting [26], which is the dosage-related specific expression of genes from either maternal or paternal origin, inactivation of the X chromosome in females [27], and activating and repressing certain genes over the course of development [28].

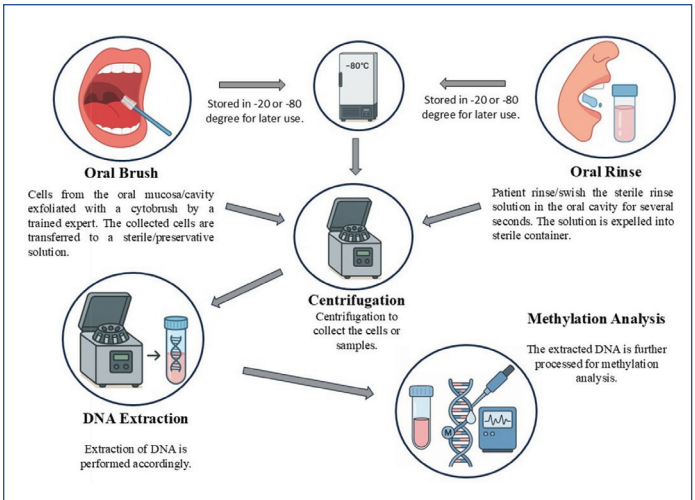
Cancer cells exhibit peculiar characteristics that largely differ from normal cells. Most cellular processes in cancer cells are dysregulated and channelled towards the rapid proliferation of cells. DNA methylation is no exception from the effects of these phenomena. In cancer cells, many studies have shown aberrant methylation compared to normal cells [29]. Intriguingly, DNA methylation dysregulation also occurs early during tumourigenesis and progresses in conjunction with cancer severity [30]. The aberrant DNA methylation patterns seen in cancer include hypermethylation, which is methylation more than normal, and hypomethylation, which is methylation less than normal. Hypermethylation usually results in repression of genes by inhibiting the transcription of genes, while hypomethylation enables and promotes gene expression [31]. Hypermethylation of genes crucial in mitigating cancer progression, such as TSGs, DNA repair genes, apoptotic genes, cell-adhesion genes, and cell cycle genes is a common phenomenon in cancer [32]. Hypermethylation of genes like MGMT, APC, and DAPK1, has been associated with cancer development [33,34]. In contrast, hypomethylation results in overexpression or upregulation of genes such as oncogenes, causes chromosomal instability, aneuploidy, and promotes tumourigenesis [31]. Hypomethylation of cancer-testis genes (germline-specific genes) such as SSX and CT-GABRA3 is reported to promote cancer progression [35]. In colorectal cancer, Long Interspersed Nucleotide Element-1 (LINE-1) hypomethylation was associated with increased risk of developing the cancer [36].

The OSCC is no exception from the aberrations in DNA methylation. Meta-analyses and cohort studies report promoter hypermethylation of TSG in 40-70% of OSCC cases, supporting its biological and clinical relevance [7]. Genes that are frequently studied for its methylation status in OSCC-such as p16, *MGMT, DAPK1, APC*, and Ras Association Domain Family Member 1 Isoform A (RASSF1A) are involved in cell-cycle control, DNA repair, apoptosis, and epithelial differentiation, pathways consistently disrupted by tobacco, areca nut, and alcohol exposure [32]. A systematic study by Don KR et al., reported prevalence of hypermethylation of p16 (43%), *DAPK* (39.7%) and *MGMT* (39.8%) in OSCC indicating that these genes are frequently methylated [37]. OSCC also parallelly exhibits global

and locus specific hypomethylation; *LINE-1* hypomethylation was detected in OSCC tissue and correlated with genomic instability and aggressive disease behaviour [38].

Oral Brush and Salivary Rinse as a Sample Source

The most trustworthy technique for determining oral cancer is tissue biopsy, an intrusive operation that may have adverse effects on patients [39]. Alternatively, an oral brush biopsy, which is non-invasive involves the exfoliation of cells from the oral mucosa or cavity using a cytobrush by a trained expert [40]. In normal tissue, epithelial cells are tightly bound together. However, premalignant and malignant cells lose their cohesive force, making them more easily exfoliated. Additionally, cancer cells and their contents are released into saliva, the bloodstream, or other bodily fluids as circulating tumour DNA (ctDNA) [41]. This presents an opportunity to use salivary rinse as another non-invasive method, which can be done by collecting saliva using a sterile rinse solution. Though the methods of sampling varies, generally patients swish the solution in their mouth for several seconds before expelling it into a sterile container for further laboratory analysis [42]. Following centrifugation, DNA and other biomolecules can be extracted for diagnostic purposes as shown in [Table/Fig-2] [43-45]. Unlike tissue biopsy, these methods are painless, minimally invasive, and can be performed repeatedly for both diagnosis and research [20].



[Table/Fig-2]: Non-invasive sampling procedure for oral cancer [43-45].

Techniques for Detection of DNA Methylation

Most common technique to detect DNA methylation is the bisulfite modification method. In this method, the DNA is first treated with sodium bisulfite which converts all the unmethylated cytosines to uracil while the methylated cytosines remain unchanged. The bisulfite-modified DNA is then subjected to various downstream detection methods to detect and analyse DNA methylation.

Whole-genome bisulfite sequencing: This technique checks for methylations throughout the entire genome. After bisulfite treatment, the treated DNA is sequenced at single-base resolution, giving genome-wide methylation profiles [46].

Reduced Representation Bisulfite Sequencing (RRBS): This technique can be used to check methylation levels at specific regions of the genome. First, restriction enzymes digest all the DNA samples and then bisulfite treated and sequenced using Next-Generation Sequencing (NGS) [47].

Methylation-Specific PCR (MSP): This method can be used to detect CpG methylations at specific loci or genes of interest. In this method, the treated DNA is subjected to Polymerase Chain Reaction (PCR) with primers designed for either methylated or unmethylated alleles of interest [48]. This technique is highly specific and sensitive.

Methylation-Sensitive Restriction Enzymes (MSREs): This method involves treating DNA first with restriction enzymes, which break unmethylated DNA but leave methylated DNA intact [49]. With PCR, the cleaved DNA is amplified where only the methylated DNA gets amplified. MSREs are region-specific.

Bisulfite Pyrosequencing: This technique combines bisulfite treatment with pyrosequencing. First, bisulfite is applied to the DNA, and then primers unique to the altered DNA are used for PCR amplification [50]. After which, pyrosequencing, a sequencing-by-synthesis method, detects nucleotide incorporation via enzyme-driven light emission. By measuring Cytosine-to-Thymine (C-to-T) conversions, it provides high-resolution, quantitative methylation data at CpG sites.

Methylation BeadChip Arrays: Methylation BeadChip arrays use bisulfite-treated DNA hybridised to oligonucleotide probes or bead arrays specific to CpG sites [51]. Methylation levels are determined by single-base extension and fluorescence detection, distinguishing methylated from unmethylated signals. This microarray-based method enables high-throughput, quantitative DNA methylation profiling across the genome, but limited to preselected CpG sites. One example of such array is Illumina Infinium Methylation Assay.

Combined Bisulfite Restriction Analysis (COBRA): This technique combines bisulfite treatment and restriction digestion. DNA is first subjected to bisulfite conversion, then to PCR amplification, and subsequently digested using restriction enzymes and separated in agarose gel for further analysis of the resulting fragments [52]. It is useful for analysing specific CpG sites but requires prior knowledge of restriction sites.

Methylated DNA Immunoprecipitation (MeDIP): This method involves the use of specific antibodies that have affinity to methylated cytosines. Genomic DNA is first heated and incubated with the antibodies specific to 5-methylcytosine and captured by magnetic bead separation [53]. The resulting complex can be subjected to PCR to check the methylation status, or microarray approaches and high throughput sequencing for genome wide studies.

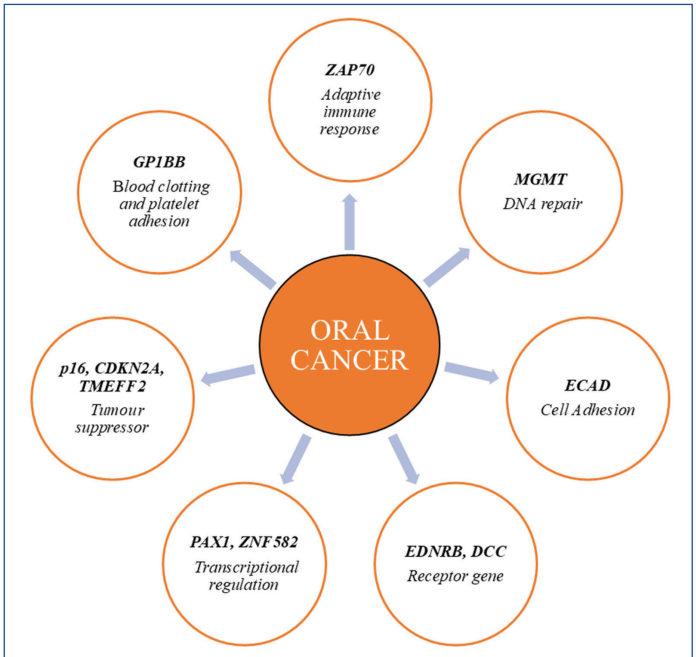
Methylated DNA in Oral Brush and Salivary Rinse as a Diagnostic and Prognostic Biomarker of Oral Cancer

Oral cancer is often diagnosed at advanced stages resulting in poor prognosis. Therefore, identifying biomarkers that would aid in early detection is crucial in mitigating the cancer fatality. Several studies have been done on the potential use of methylated DNA as biomarkers in tissues of oral cancer. The potential methylated DNA biomarkers in oral brush and salivary rinse of oral cancer and its precancerous lesions is summarised in [Table/Fig-3] [38,43-45,54-64]. The molecular function of the genes is also shown in [Table/Fig-4].

An assessment of methylation in samples from oral brush of OSCC, precancerous lesions, and normal cells using bisulfite NGS identified *ZAP70* and *GP1BB* as early potential markers [43]. In this study, *ZAP70* hypermethylation was present in 100% of OSCC and High-Grade Squamous Intraepithelial Lesions (HG-SIL), while *GP1BB* hypomethylation occurred in 90% of these cases compared to normal. In oral cancer, methylation occurs early which is reflected in studies pertaining to its precancerous lesions. Alterations in p16 methylation in OSMF compared to normal cells were explored using oral brush samples [54]. Here, 93.3% of OSMF cases showed p16 methylation, whereas no methylation was observed in normal samples. The present study also underscores the efficacy of oral brush samples in detecting methylation. Oral brushes could also be used to screen for groups thought to be at risk of acquiring oral cancer. A study by Ruesga MT et al., showed that 20% of oral brush samples from OSCC risk groups showed p16 methylation with a higher frequency observed in patients with a history of head and neck cancer [55].

Gene marker	Source	Inference	Primary utility	Reference
ZAP70 and GP1BB	oral brush	*ZAP70* hypermethylation and *GP1BB* hypomethylation could differentiate OSCC and HG-SIL from normal cells	Diagnosis	[43]
p16	oral brush and oral rinse	p16 hypermethylation could be used to screen for groups showing OSMF lesions for risk of acquiring oral cancer.	Diagnosis/Risk stratification	[54]
p16	oral brush	p16 methylation appeared in OSCC risk groups with higher frequency in patients with history of head and neck cancer.	Diagnosis/Risk stratification	[55]
ZNF582 and PAX1	oral brush & oral rinse	Increased frequency of hypermethylation correlated with OSCC severity. Hypermethylation was linked to OSCC recurrence.	Screening / Prognosis	[56,64]
Thirteen-genes panel	oral brush	Methylation positive in OSCC and OL compared to normal. Methylation was associated with local regional relapse, hence a potential marker for prognosis. It could also serve as a marker for high-risk individuals as all dysplastic OL showed methylation. Consistent methylation score from OL to OSCC, to primary resection, except following secondary OSCC resection (disease free)	Prognosis / Monitoring	[44,57,58]
p16 and MGMT	oral rinse	Significantly hypermethylated in OL compared to normal	Diagnosis/Screening	[59]
LINE-1	oral rinse	Hypomethylation detected in both salivary rinse and OSCC tissue compared to normal with same efficiency.	Screening /Risk	[38]
ECAD, TMEFF2, RARβ, and MGMT	oral rinse	Methylation of these genes had the potential to distinguish OSCC from normal cells with a high sensitivity and specificity (>75%) and Area Under the Curve (AUC) values greater than 80%	Diagnosis/Screening	[45]
EDNRB and DCC	oral rinse	Methylation of these genes could help differentiate malignant from non-malignant lesions in OSCC	Diagnosis/Screening	[60]
CDKN2A	oral rinse	Promoter methylation of *CDKN2A* was detected in OSCC while none was detected in normal samples.	Diagnosis/Screening	[61]
7 CpG loci	oral rinse	These CpG sites methylation was linked to patient survival.	Prognosis	[62]
22 CpG sites	oral rinse	Methylation of these CpG sites showed high accuracy in predicting oral cancer, underscoring its potential for early screening	Screening	[63]

[Table/Fig-3]: Potential diagnostic and prognostic biomarkers in oral brush/rinse of OSCC and its precancerous lesions [38,43-45,54-64].
HG-SIL: High-Grade Squamous Intraepithelial Lesion



[Table/Fig-4]: The molecular functions of the potential methylated DNA biomarkers in oral cancer detected from oral brush and rinses.

ZNF582 and *PAX1* hypermethylation in oral brush samples from OED and OSCC helped distinguish between mild and moderate dysplasia, with increased methylation correlating with OSCC severity [56]. These genes' hypermethylation was also connected to oral cancer recurrence, indicating their potential as biomarkers for disease monitoring and recurrence.

Thirteen genes panel (ZAP70, ITGA4, KIF1A, PARP15, EPHX3, NTM, LRRTM1, FLI1, MIR193, LINC00599, MIR296, TERT, and GP1BB) and their prognostic potential in oral brush of OSCC samples was shown in a study by Gissi DB et al., 95.9% of the samples showed positive results for methylation compared to normal. Six months after OSCC resection, 16 oral brush samples from the regenerative area tested positive, with six of these cases experiencing local regional relapse, hence highlighting its prognostic potential [44]. Another study revealed differential methylation of the same gene panel in OL and OSCC compared to healthy individuals; all dysplastic OL samples showed positive scores, highlighting the potential of this gene panel in predicting high-risk individuals for OSCC [65]. The 13-gene panel was subsequently validated in an Italian cohort of 220 oral brush samples, where it detected 93.6% of OSCC cases and correctly identified 84.9% of normal samples as negative [57]. However, the present study did not evaluate the prognostic value of the panel.

The prognostic value of the 13-gene panel, with a calculated threshold score of 1.061554, was evaluated in a single patient over five intervals: during OL diagnosis, primary OSCC diagnosis, six months post-primary OSCC resection, secondary OSCC emergence, and six months post-secondary OSCC resection [58]. The test scores surpassed the threshold and were positive for OL (1.61), primary OSCC (5.21), six months post-primary OSCC resection (1.85), and secondary OSCC (8.14). However, six months after secondary OSCC resection, the score dropped below the threshold to 0.47, indicating a negative result, with the patient being disease-free. This study demonstrated that the methylation score remained consistently positive throughout the sampling intervals, except following secondary OSCC resection. Therefore, DNA methylation analysis using this gene panel and the oral brush technique shows strong potential as a prognostic tool for cancer management. Nonetheless, the present small sample size necessitates further validation in larger cohorts.

A study on p16 methylation in OSMF found that oral brush samples were more efficient in detecting methylation than salivary rinses. In this study, 93% of OSMF brush samples and 50% of salivary rinse samples showed p16 methylation [54]. Similarly, another study demonstrated that tissue and oral swabs had comparable efficiency in assessing DNA methylation in both cancerous and normal tissues [66]. Based on these findings, oral brush and salivary samples can serve as non-invasive source for detecting DNA methylation and act as early diagnostic tools for cancer, with oral brush samples being notably more sensitive.

Salivary/oral rinse has the potential to detect gene methylation associated with oral cancer. A study has shown that oral rinse is

an efficient alternative to matched tissue samples for identifying the methylation of certain genes implicated in oral cancer through quantitative MSP [67]. Several genes methylation has been detected in oral rinses of precancerous and cancerous lesions using the MSP technique. p16 and *MGMT* was found to be significantly hypermethylated in OL compared to normal [59]. *LINE-1* hypomethylation was also detected in both the oral rinses and tissue of OSCC patient compared to normal. Moreover, oral rinse showed the same efficiency of detecting *LINE-1* methylation as compared to OSCC tissue [38]. A study by Nagata S et al., has demonstrated that methylation differences off four genes (*ECAD, TMEFF2,RAR*β, and *MGMT*) in oral rinses of 34 OSCC and 24 control samples have the potential to distinguish cancer from normal cells. These four genes had high sensitivity and specificity (>75%) and AUC values greater than 80% in detecting OSCC, thereby suggesting its potential as a cancer biomarker using oral rinses [45]. Additionally, a study showed that OSCC can be differentiated from normal cells based on variations in the methylation of genes found in oral rinses. For example, hypermethylation of *EDNRB* and *DCC* was detectable in salivary rinses and could help differentiate malignant from non-malignant lesions in OSCC [60].

Fourty percent of OSCC patients in a preliminary study showed promoter methylation of *CDKN2A* in oral rinses, while all healthy individuals had no methylation [61]. Additionally, Langevin SM et al., identified seven CpG loci (cg02319972, cg03784083, cg15740054, cg18928362, cg21022792, cg21702497, cg25914931) in oral rinse samples of OSCC, which were associated with survival, but only one locus (cg21022792), corresponding to *GABBR1*, was validated in a separate OSCC cohort [62]. A methylation classifier comprising 22 CpG islands in oral rinses showed high accuracy in predicting oral cancer, underscoring its potential for early screening [63].

In the salivary rinse of OSMF, p16 methylation showed a 50% positive rate, while all the normal samples were negative [54]. Previous studies had highlighted the potential of *ZNF582* and *PAX1* methylation as biomarkers for OED and oral cancer recurrence using oral brush samples [56]. Similarly, in oral rinses, both *ZNF582* and *PAX1* showed similar results, although oral brush samples was more efficient in terms of sensitivity and accuracy [64]. Overall, p16 methylation positivity shows a wide range across studies (approximately 50-93%). This variation is expected and likely reflects differences in sample type, lesion severity, comparison groups, and the methylation detection methods used across studies.

In clinical practice, methylation-based biomarkers detected in oral brush and salivary rinse samples should be regarded as adjunctive tools rather than replacements for histopathological diagnosis. A negative methylation test result indicating the absence of detectable methylation in the sampled material should be interpreted with caution and does not necessarily rule out early or low-burden disease. While these assays often demonstrate a high positive predictive value, their negative predictive value may be lower, particularly in early lesions, low-grade dysplasia, or samples with limited epithelial DNA. Accordingly, a negative result should not override clinical judgment or replace careful oral examination and biopsy when clinical suspicion persists. Oral brush–based assays generally show higher sensitivity for detecting OSCC and high-grade dysplasia, reflecting their enriched epithelial content and lesion-specific epigenetic signals, and are therefore more suitable for diagnostic confirmation and risk stratification [43,57]. In contrast, salivary and oral rinse based assays offer moderate sensitivity but greater feasibility for large-scale screening and surveillance, capturing exfoliated tumour DNA and field cancerisation effects, with reported diagnostic accuracies of approximately 75-85% for selected gene panels [60,63]. For prognostic use, longitudinal assessment of multi-gene methylation panels, particularly oral brush- based panels, has shown associations with recurrence and disease-free survival, supporting their potential role in post-treatment monitoring [44,58].

Given the biological heterogeneity of oral cancer, combining multiple methylation markers into gene panels improves diagnostic and prognostic performance compared with single-gene assays [44,65,57,63]. However, interpretation of these biomarkers may be influenced by lifestyle factors, oral inflammation, microbial changes, and technical variability, which can affect specificity [68,69]. Consequently, standardised protocols, careful patient stratification, and rigorous analytical and clinical validation along with regulatory approval are essential before routine clinical implementation [68,70].

Challenges and Limitations

Using methylated DNA biomarkers for the early detection of oral cancer through non-invasive methods like oral brush and salivary rinse also faces limitations in clinical use. There is a lack of standardisation in sample collection, processing and analysis across studies which could result in variation [68]. This will affect reproducibility as result of differences that could arise from DNA extraction, bisulfite conversion and detection methods. Salivary rinse may also contain a mixture of host and microbial DNA, unlike oral brush which contains richer epithelial content resulting in higher efficiency. Several promising biomarkers panels have remained unvalidated in large, diverse cohorts due to small sample sizes and patient participation factor. Another challenge is the presence of biological and environmental factors such as oral inflammation, infections, microbiota shifts, and lifestyle habits like tobacco and alcohol use, that complicate methylation interpretation [69]. Through persistent inflammatory signalling and oxidative stress, oral inflammation, periodontal disease, microbial dysbiosis, and local infections can affect DNA methylation patterns in the mouth epithelium. Low-level, gene-specific methylation changes have been linked to these disorders; however, these changes are typically less frequent and do not primarily affect TSGs, and are less stable than methylation events linked to cancer [71]. However, these methylation changes due to inflammatory factors mostly affect specificity rather than sensitivity in methylation-based diagnostic investigations [68,71]. The exact magnitude of this effect on diagnostic accuracy has not yet been precisely quantified in large, prospective OSCC cohorts. Crucially, these effects are greatly reduced by the use of multi-gene methylation panels maintaining overall diagnostic performance for OSCC detection [57,58].

Also, most studies are retrospective or cross-sectional. This could limit insights into how methylation changes with disease progression or treatment. The high cost of advanced methylation detection is also another factor hindering widespread adoption, especially in low-resource areas with high oral cancer rates. However, from a healthcare delivery perspective, oral brush and salivary methylation assays are significantly more affordable per test than scalpel biopsies. While a traditional biopsy requires surgical consumables, clinician time, and labour-intensive histopathology [72], non-invasive molecular sampling utilises standardised collection kits and high-throughput processing. Economic evaluations suggest these assays are most effective as “gatekeeper” tools. By using stable methylation markers to triage patients, clinicians can prioritise those truly needing surgery while sparing low-risk cases from unnecessary invasive procedures and the associated productivity loss [73]. Ultimately, these assays serve as cost-effective surveillance tools that optimise specialised surgical resources.

In addition, the use of liquid biopsy is another transformative method in oncology. However, its integration with oral cancer diagnosis remains underexplored. Research should focus in combining local and methylated DNA biomarkers in diagnosing oral cancer to enhance early detection and disease monitoring strategies.

Future Directions

Future directions should focus on addressing the current challenges to maximise the potential of methylated DNA biomarkers in oral brush and salivary samples. Population-specific, large-scale, multicentre, and prospective studies are required to validate the potential and promising panels of methylated DNA. Standardising the protocols for handling sample, DNA extraction and detection will also greatly improve the reproducibility and support broader clinical use [70]. At the same time, expanding the current studies on methylome analysis could also help uncover highly specific new markers. These complex methylation patterns could be incorporated into algorithms for machine learning to create precise, predictive diagnostic tools.

Another approach that could revolutionised diagnosis is the combination of oral-based sampling with plasma-derived liquid biopsy strategies. Detecting ctDNA and circulating free DNA (cfDNA) in blood alongside oral or salivary rinse samples could provide a dual-modality method that may greatly improve the specificity and sensitivity of diagnosis. Several studies have shown the feasibility of this strategy in several cancers [74]. To enhance prognosis and personalised therapy, longitudinal research is also critical to track methylation changes over the course of disease and treatment. To bring the methylated DNA biomarkers into clinical use, there is an urgent need for affordable, rapid, and portable methylation detection assays- especially in low-resource settings to enable early detection in high-risk groups.

CONCLUSION(S)

The rise in oral cancer cases underscores the need for more effective and patient-friendly diagnostic approaches. While traditional biopsies remain the gold standard, it comes with a limitation due to their invasive nature often limiting early detection. In the present review, DNA methylation has emerged as a useful biomarker due to its role in early tumourigenesis and disease progression. Non-invasive methods like oral brush biopsies and salivary rinses offer a practical alternative for detecting these epigenetic changes, providing a less invasive way to screen high-risk individuals. These methods might also motivate regular monitoring and follow-up, which are crucial for early intervention and better clinical outcomes. Although further research and validation are needed to fully integrate these tools into clinical practice, they represent a promising step toward more accessible and personalised oral cancer diagnostics. Embracing such advancements could ultimately lead to earlier diagnoses, improved patient compliance, and improved public health tactics in cancer care.

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