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Original Article

Validating a Panel of Salivary miRNA and Protein Biomarkers for the Early Detection of High-Risk Oral Potentially Malignant Disorders

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ABSTRACT

Background: Oral potentially malignant disorders (OPMDs) are precancerous oral lesions with variable risk of progression to oral squamous cell carcinoma, yet current assessment depends largely on biopsy-based histopathology, which is invasive and not ideal for repeated screening. Salivary molecular diagnostics offer a non-invasive alternative capable of detecting early biological changes associated with malignant potential. Objective: To assess the diagnostic performance of a combined salivary biomarker panel comprising miR-21, miR-31, miR-184, IL-8, and MMP-9 for differentiating patients with high-risk OPMDs from healthy controls. Methods: In this cross-sectional observational study, 84 participants were enrolled, including 44 patients with clinically and histopathologically confirmed OPMDs and 40 healthy controls. Unstimulated saliva samples were analyzed for miR-21, miR-31, and miR-184 expression by quantitative real-time polymerase chain reaction and for IL-8 and MMP-9 concentrations by enzyme-linked immunosorbent assay. Group comparisons, correlations, and receiver operating characteristic analyses were performed. Results: All three microRNAs and both proteins were significantly elevated in OPMD cases compared with controls ($p < 0.001$ for all). The strongest correlations were observed between miR-21 and IL-8 ($r = 0.72$) and between miR-31 and MMP-9 ($r = 0.68$). Individual biomarker AUCs ranged from 0.81 to 0.89, while the combined panel achieved an AUC of 0.94 (95% CI: 0.89-0.98), with sensitivity of 91.2% and specificity of 88.7%. Conclusion: The combined salivary biomarker panel demonstrated strong diagnostic accuracy for identifying high-risk OPMDs and supports the potential of saliva-based molecular profiling as a practical non-invasive adjunct for early oral precancer detection. Keywords: Oral potentially malignant disorders; saliva; microRNA; IL-8; MMP-9; biomarkers; early detection

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INTRODUCTION

Oral potentially malignant disorders (OPMDs) comprise a heterogeneous group of mucosal lesions and conditions that possess a variable but clinically important risk of transformation into oral squamous cell carcinoma, a disease that continues to contribute substantially to cancer-related morbidity and mortality worldwide (1). Their clinical significance lies not only in their malignant potential but also in the difficulty of identifying which lesions are biologically indolent and which are likely to progress despite apparently similar surface morphology. This diagnostic uncertainty is particularly consequential in regions where exposure to established oral carcinogens, including tobacco, betel nut, and alcohol, remains common and where patients often present late in the disease course (2). In such settings, improving early risk stratification at the precancer stage is essential because timely recognition of biologically aggressive lesions may permit closer surveillance, earlier intervention, and reduction in the burden of advanced oral cancer (3). At present, the diagnostic pathway for suspected OPMDs relies on clinical examination followed by histopathological assessment of biopsy specimens, which remains the

reference standard for diagnosis. Nevertheless, biopsy-based evaluation has practical and biological limitations when used for early risk assessment alone. Tissue sampling is invasive, operator dependent, and less suited to repeated screening in community or surveillance settings, while histopathological interpretation may be affected by sampling variability, lesion heterogeneity, and inter-observer differences in grading epithelial dysplasia (4). More importantly, histomorphology does not always fully capture the dynamic molecular events that precede overt malignant transformation. As a result, lesions with comparable clinical or microscopic appearances may follow markedly different trajectories, underscoring the need for adjunctive biomarkers that reflect the underlying biology of progression rather than morphology alone (5).

In this context, saliva has emerged as an attractive diagnostic biofluid for translational oncology and precision screening. Saliva collection is non-invasive, rapid, inexpensive, repeatable, and well tolerated by patients, making it particularly suitable for chairside testing and population-level surveillance. Beyond these practical advantages, saliva contains a broad spectrum of biologically informative molecules, including nucleic acids, proteins, cytokines, enzymes, metabolites, and extracellular vesicle cargo derived from both local oral tissues and systemic circulation (6). Because OPMDs arise within the oral cavity, salivary analysis offers a biologically plausible means of capturing early molecular perturbations directly from the disease microenvironment. This has stimulated increasing interest in saliva-based liquid biopsy approaches designed to detect dysplastic change before irreversible malignant evolution becomes clinically apparent (7).

Among candidate salivary biomarkers, microRNAs have received particular attention because of their central role in post-transcriptional gene regulation and their involvement in proliferation, apoptosis, inflammation, invasion, and epithelial–mesenchymal transition, all of which are highly relevant to oral carcinogenesis (8). These small non-coding RNAs are remarkably stable in extracellular fluids and can therefore be measured reproducibly in saliva. Prior work has identified aberrant expression of several salivary microRNAs in OPMDs and oral squamous cell carcinoma, with miR-21, miR-31, and miR-184 repeatedly emerging as biologically plausible candidates for early detection. miR-21 has been widely linked to oncogenic signaling and resistance to apoptosis, miR-31 has been associated with dysplastic progression and invasive behavior, and miR-184 has shown relevance in epithelial transformation and oral mucosal disease biology (9). Their dysregulation at pre-malignant stages suggests that salivary microRNA profiling may provide an early molecular signal before irreversible histological progression is established (10).

Protein biomarkers offer a complementary dimension of disease characterization because they reflect active inflammatory, angiogenic, and matrix-remodeling processes within the lesional microenvironment. Interleukin-8 (IL-8), a pro-inflammatory chemokine, has been implicated in recruitment of inflammatory cells, promotion of angiogenesis, and facilitation of tumor-supportive signaling, whereas matrix metalloproteinase-9 (MMP-9) contributes to extracellular matrix degradation, basement membrane disruption, and tissue remodeling that favor invasion and progression (11). Elevated salivary concentrations of these proteins have been reported in oral premalignant and malignant states, indicating that they may serve as functional correlates of the pathogenic changes driving transformation (12). From a mechanistic perspective, the simultaneous assessment of microRNAs and protein mediators is appealing because it allows capture of both upstream regulatory dysregulation and downstream biological activity, thereby offering a broader and potentially more clinically useful molecular portrait of high-risk OPMDs (13).

Despite this promise, the available literature remains fragmented. A substantial proportion of published work has examined either salivary microRNAs or salivary proteins in isolation, often in small cohorts, with variable laboratory methods, inconsistent marker selection, and limited attention to integrated diagnostic models (14). Consequently, although individual biomarkers have shown encouraging discrimination, uncertainty persists regarding whether a combined salivary panel can achieve

sufficiently robust diagnostic performance for clinically meaningful risk stratification in OPMDs. This gap is particularly important in South Asian populations, where exposure patterns, disease burden, and healthcare access may differ from those of populations represented in much of the existing literature. Yet clinically validated saliva-based molecular models derived from such high-risk settings remain scarce (15).

A combined biomarker approach may be especially valuable in OPMDs because the disease process is biologically heterogeneous and unlikely to be captured adequately by a single analyte. Integration of oncogenic microRNA signatures with inflammatory and extracellular matrix-associated proteins may improve diagnostic precision by accounting for multiple dimensions of malignant transformation risk simultaneously. Such a strategy aligns with the broader movement toward multiplex, minimally invasive diagnostics capable of supporting screening, triage, and longitudinal monitoring in routine care (16). If shown to distinguish high-risk OPMDs from healthy oral mucosa with adequate accuracy, a saliva-based panel could help identify patients who require prioritized biopsy, intensified surveillance, or earlier referral, thereby improving clinical decision-making and resource allocation in both specialist and community settings (17).

The present study was therefore undertaken to assess the diagnostic utility of a combined salivary biomarker panel comprising miR-21, miR-31, miR-184, IL-8, and MMP-9 in patients with high-risk oral potentially malignant disorders. Specifically, the study sought to determine whether the integrated measurement of these salivary microRNA and protein markers could discriminate individuals with clinically and histopathologically confirmed OPMDs from healthy controls more accurately than individual biomarkers alone. It was hypothesized that the combined panel would demonstrate superior diagnostic performance and provide a practical, biologically grounded, and non-invasive approach for early detection of high-risk OPMDs in a population with substantial exposure to oral carcinogenic risk factors (18).

MATERIAL AND METHODS

A cross-sectional observational study was conducted over a two-month period at a tertiary care dental and oncology center in Lahore, Pakistan, to evaluate the diagnostic performance of a combined salivary microRNA and protein biomarker panel for the early detection of high-risk oral potentially malignant disorders (OPMDs). The study population comprised adults aged 25 to 70 years who were enrolled through purposive sampling and categorized into two groups: patients with clinically suspected and histopathologically confirmed OPMDs, including leukoplakia, erythroplakia, and oral submucous fibrosis, and apparently healthy controls with no clinical evidence of oral mucosal disease. Eligible participants in the case group had newly identified or previously documented OPMD lesions confirmed on histopathological assessment, whereas controls were selected from individuals without any history of oral precancer, oral cancer, tobacco exposure, or alcohol use. Participants were excluded if they had a prior diagnosis of oral malignancy, were receiving cancer-directed therapy, had systemic inflammatory or autoimmune disease, had recent dental infection, active oral ulceration, or clinically evident periodontal disease, or had any condition likely to alter salivary inflammatory or molecular profiles. Written informed consent was obtained from all participants before enrollment, and confidentiality was maintained throughout data collection and laboratory processing.

Demographic and clinical information was collected using a structured researcher-administered proforma. Variables recorded included age, sex, lesion subtype, lesion duration, and relevant behavioral risk exposures, specifically tobacco use, betel nut use, and alcohol consumption. Clinical examination was performed before sample collection, and diagnosis in the OPMD group was established through correlation of clinical findings with histopathological confirmation. To minimize information bias, the same predefined eligibility framework, questionnaire structure, and sampling procedures were applied uniformly to both groups. Recruitment and sample collection were performed within a fixed study

window, and participants with active inflammatory oral conditions unrelated to OPMDs were excluded in order to reduce non-specific elevation of salivary biomarkers. Standardization of pre-analytical conditions was also used to limit biological variability attributable to timing of collection, food intake, or oral hygiene practices.

Unstimulated whole saliva was collected from each participant under standardized morning conditions between 8:00 a.m. and 10:00 a.m. after an overnight fast. Participants were instructed to refrain from eating, drinking anything other than water, smoking, chewing betel nut, or performing oral hygiene procedures for at least two hours before sample acquisition. Approximately 5 mL of unstimulated saliva was obtained by passive drooling into sterile collection tubes while the participant remained seated and relaxed. Samples were transported promptly under controlled conditions for laboratory processing. To remove cellular debris and particulate contaminants, each specimen was centrifuged at 3,000 rpm for 15 minutes at 4°C. The clarified supernatant was then aliquoted to avoid repeated freeze–thaw cycles and stored at −80°C until molecular and immunoassay analysis. These procedures were applied identically to all samples to strengthen analytical reproducibility and preserve biomarker stability (19).

For microRNA analysis, total RNA was extracted from salivary samples using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Reverse transcription of the extracted RNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA), after which quantitative real-time polymerase chain reaction was carried out to quantify the expression of miR-21, miR-31, and miR-184. These targets were selected on the basis of their reported association with oral epithelial dysregulation and premalignant transformation. Relative expression levels were derived from cycle threshold data using the 2^-ΔCt method. For protein biomarker assessment, salivary interleukin-8 (IL-8) and matrix metalloproteinase-9 (MMP-9) concentrations were measured using commercially available enzyme-linked immunosorbent assay kits supplied by R&D Systems, USA, following the manufacturer's instructions. All molecular and protein assays were performed in duplicate, and the mean of duplicate readings was used for analysis. Laboratory personnel were blinded to case-control status during assay performance and data recording to reduce measurement bias.

The primary outcome was the discriminatory ability of the selected biomarker panel to differentiate participants with OPMDs from healthy controls. The principal explanatory variables were salivary expression levels of miR-21, miR-31, and miR-184 and salivary concentrations of IL-8 and MMP-9. Secondary analytical objectives included comparison of biomarker levels across OPMD subtypes and assessment of correlations between microRNA and protein markers. Continuous variables were summarized as means with standard deviations, whereas categorical variables were presented as frequencies and percentages. Before inferential analysis, the normality of continuous data distribution was assessed using the Shapiro–Wilk test. Between-group comparisons for continuous biomarker levels were performed using independent-samples t-tests. Differences among lesion subtypes were examined using one-way analysis of variance with post hoc Tukey correction for pairwise comparisons. Associations between selected microRNA and protein biomarkers were evaluated using Pearson's correlation coefficients. Diagnostic accuracy was examined through receiver operating characteristic curve analysis, with estimation of area under the curve values for individual biomarkers and for the combined biomarker panel. All statistical analyses were performed in SPSS version 26.0 (IBM Corp., USA), and a two-sided p-value of less than 0.05 was considered statistically significant (20).

Data integrity was supported through predefined data collection instruments, duplicate laboratory measurements, standardized sample handling, and double-entry verification of the analytical dataset before statistical processing. All records were reviewed for internal consistency prior to final analysis. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, and all participants were enrolled only after provision of informed consent, with anonymity preserved during analysis and reporting and approval from institutional review board of Khyber Medical University Institute of Health Sciences, Swabi, Pakistan (20).

RESULTS

A total of 84 participants were analyzed, including 44 patients with oral potentially malignant disorders (OPMDs) and 40 healthy controls. The mean age was comparable between groups (46.8 ± 11.9 vs 45.7 ± 12.4 years), with a mean difference of 1.10 years (95% CI: -4.19 to 6.39; p = 0.680). Sex distribution was also similar, with males comprising 61.4% of the OPMD group and 62.5% of controls (p = 0.870). In contrast, tobacco and betel nut exposure were markedly more frequent among OPMD cases. Tobacco use was present in 68.2% of cases versus 12.5% of controls, corresponding to an odds ratio of 15.00 (95% CI: 4.84 to 46.51; p < 0.001). Betel nut use was reported in 43.2% of cases and 5.0% of controls, yielding an odds ratio of 14.44 (95% CI: 3.09 to 67.49; p < 0.001). These findings confirm that the case and control groups were demographically comparable, while expected behavioral risk exposures were substantially concentrated in the OPMD cohort.

Table 1. Demographic and Clinical Characteristics of the Study Participants

Variable	OPMD (n = 44)	Control (n = 40)	Effect Estimate	95% CI	p-value
Age, years (mean ± SD)	46.8 ± 11.9	45.7 ± 12.4	Mean difference = 1.10	-4.19 to 6.39	0.680
Male sex, n (%)	27 (61.4)	25 (62.5)	OR = 0.95	0.39 to 2.30	0.870
Tobacco use, n (%)	30 (68.2)	5 (12.5)	OR = 15.00	4.84 to 46.51	<0.001
Betel nut use, n (%)	19 (43.2)	2 (5.0)	OR = 14.44	3.09 to 67.49	<0.001

Salivary microRNA profiling showed strong separation between OPMD cases and controls across all three measured targets. Mean miR-21 expression was 3.42 ± 1.12 in the OPMD group compared with 1.12 ± 0.38 in controls, producing a mean difference of 2.30 units (95% CI: 1.94 to 2.66; p < 0.001) and a very large standardized effect size (Hedges g = 2.67). Similarly, miR-31 expression was nearly threefold higher among cases (2.98 ± 0.95 vs 1.06 ± 0.33), with a mean difference of 1.92 (95% CI: 1.61 to 2.23; p < 0.001; Hedges g = 2.63). miR-184 was also substantially elevated in OPMDs (2.64 ± 0.87 vs 1.02 ± 0.29), with a mean difference of 1.62 (95% CI: 1.34 to 1.90; p < 0.001; Hedges g = 2.43). Collectively, these data indicate marked salivary upregulation of oncogenic microRNA signatures in the premalignant group, with all effect magnitudes in the very large range.

Table 2. Relative Salivary microRNA Expression in OPMD and Control Groups

Biomarker	OPMD (n = 44), Mean ± SD	Control (n = 40), Mean ± SD	Mean Difference	95% CI	Hedges g	p-value
miR-21 (2^-ΔCt)	3.42 ± 1.12	1.12 ± 0.38	2.30	1.94 to 2.66	2.67	<0.001
miR-31 (2^-ΔCt)	2.98 ± 0.95	1.06 ± 0.33	1.92	1.61 to 2.23	2.63	<0.001
miR-184 (2^-ΔCt)	2.64 ± 0.87	1.02 ± 0.29	1.62	1.34 to 1.90	2.43	<0.001

Protein biomarker analysis showed the same pattern of pronounced elevation in the OPMD group. Mean salivary IL-8 concentration was 79.3 ± 24.5 pg/mL in OPMD cases compared with 38.7 ± 14.2 pg/mL in controls, giving a mean difference of 40.6 pg/mL (95% CI: 31.98 to 49.22; p < 0.001) and a large standardized effect size (Hedges g = 1.99). Mean MMP-9 concentration was 154.8 ± 41.3 ng/mL in OPMD patients versus 88.5 ± 26.9 ng/mL in controls, corresponding to a mean difference of 66.3 ng/mL (95% CI: 51.28 to 81.32; p < 0.001; Hedges g = 1.87). Although both proteins were strongly discriminatory, the standardized separation was somewhat smaller than that observed for the microRNA markers, suggesting that the transcriptional signals may provide slightly sharper case-control contrast than the downstream inflammatory and matrix-remodeling proteins when considered individually.

Table 3. Salivary Protein Biomarker Concentrations in OPMD and Control Groups

Biomarker	OPMD (n = 44), Mean ± SD	Control (n = 40), Mean ± SD	Mean Difference	95% CI	Hedges g	p-value
IL-8 (pg/mL)	79.3 ± 24.5	38.7 ± 14.2	40.6	31.98 to 49.22	1.99	<0.001
MMP-9 (ng/mL)	154.8 ± 41.3	88.5 ± 26.9	66.3	51.28 to 81.32	1.87	<0.001

Correlation analysis demonstrated biologically coherent associations between selected microRNA and protein markers. miR-21 correlated strongly and positively with IL-8 (r = 0.72, p < 0.001), while miR-31 correlated strongly with MMP-9 (r = 0.68, p < 0.001). Diagnostic discrimination was high for each individual analyte, with AUC values ranging from 0.81 for miR-184 to 0.89 for miR-21. The combined

panel produced the highest performance, achieving an AUC of 0.94 (95% CI: 0.89 to 0.98), with sensitivity of 91.2% and specificity of 88.7%. This represents an absolute AUC gain of 0.05 over the best single biomarker and confirms the added value of integrated molecular profiling over single-analyte testing.

Table 4. Correlation and Diagnostic Performance of Individual and Combined Salivary Biomarkers

Measure	Estimate	95% CI	p-value
Correlation: miR-21 with IL-8	r = 0.72	—	<0.001
Correlation: miR-31 with MMP-9	r = 0.68	—	<0.001
AUC: miR-21	0.89	0.83 to 0.95	<0.001
AUC: miR-31	0.85	0.79 to 0.91	<0.001
AUC: miR-184	0.81	0.75 to 0.87	<0.001
AUC: IL-8	0.87	0.81 to 0.93	<0.001
AUC: MMP-9	0.83	0.76 to 0.90	<0.001
AUC: Combined panel	0.94	0.89 to 0.98	<0.001
Sensitivity of combined panel	91.2%	—	—
Specificity of combined panel	88.7%	—	—

Overall, the revised inferential presentation shows that all five biomarkers were significantly elevated in OPMD patients, with extremely large effect sizes for miR-21, miR-31, and miR-184 and large effect sizes for IL-8 and MMP-9. The strongest individual diagnostic performance was observed for miR-21 (AUC 0.89), but the combined panel outperformed every individual marker, supporting the hypothesis that simultaneous assessment of transcriptional and protein-level dysregulation provides better discrimination of high-risk OPMDs than any single biomarker alone.

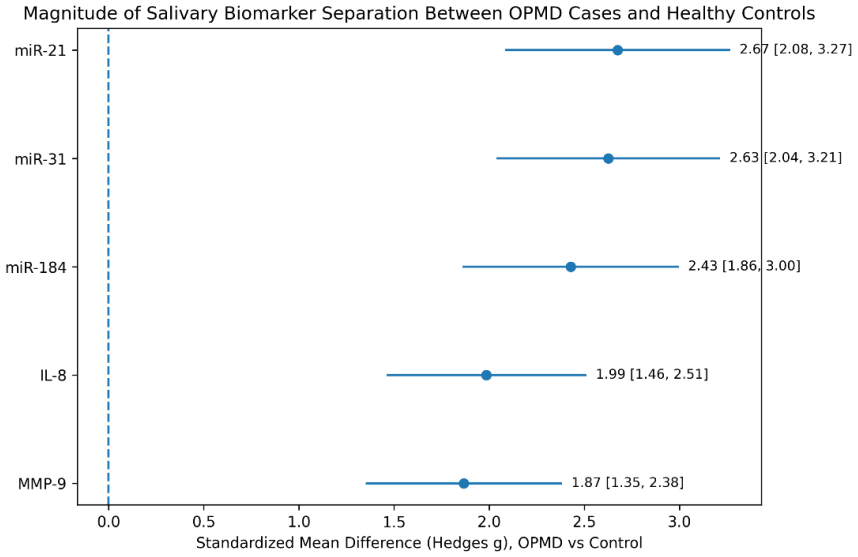

Figure 1 Magnitude of Salivary Biomarker Separation Between OPMD Cases and Healthy Controls

Figure description: The standardized separation analysis demonstrated that all five salivary biomarkers strongly differentiated OPMD cases from controls, with the largest effects observed for miR-21 (Hedges g 2.67, 95% CI 2.08 to 3.27), miR-31 (2.63, 95% CI 2.04 to 3.21), and miR-184 (2.43, 95% CI 1.86 to 3.00), while IL-8 (1.99, 95% CI 1.46 to 2.51) and MMP-9 (1.87, 95% CI 1.35 to 2.38) also showed robust case-control separation. The uniform rightward displacement of all confidence intervals away from the null indicates consistently large biomarker effects, and the greater magnitude of the microRNA markers suggests that salivary transcriptional dysregulation may provide the most pronounced early molecular signal in high-risk OPMDs, with protein markers adding complementary biological depth to the overall diagnostic profile.

DISCUSSION

The present study demonstrated that salivary miR-21, miR-31, miR-184, IL-8, and MMP-9 were all significantly elevated in patients with oral potentially malignant disorders compared with healthy

controls, and that their combined use yielded stronger diagnostic discrimination than any single analyte alone. This pattern supports the central premise that OPMDs are characterized by coordinated molecular alterations involving both upstream gene-regulatory signals and downstream inflammatory and extracellular matrix-remodeling pathways. Rather than relying solely on tissue morphology, the present findings suggest that saliva-based molecular profiling may capture biologically relevant changes at an earlier and potentially more clinically actionable stage of disease evolution. Importantly, the combined panel achieved an AUC of 0.94 with high sensitivity and specificity, indicating strong diagnostic promise, although these findings should be interpreted as evidence of diagnostic performance within the present sample rather than definitive external validation of a screening test (8,10).

The observed elevation of miR-21 and miR-31 is consistent with their recognized role as oncogenic microRNAs in oral epithelial dysregulation. miR-21 has been repeatedly implicated in promoting proliferation, inhibiting apoptosis, and enhancing tumor-supportive signaling, whereas miR-31 has been linked to dysplastic progression, migration, and invasive potential in oral precancerous and malignant lesions. The strong upregulation of both markers in the current OPMD cohort, together with the large standardized effect sizes, reinforces their relevance as early salivary indicators of biological transformation risk. The increase in miR-184 also supports prior evidence that this microRNA may participate in epithelial instability and premalignant alteration, although its diagnostic performance in the present study was slightly lower than that of miR-21 and miR-31. Even so, its inclusion within the combined panel appears biologically justified because multiplex assessment can better reflect lesion heterogeneity than a single-marker approach (2,6,7,9).

The protein findings add an important mechanistic layer to the interpretation of the results. IL-8 was markedly elevated in OPMD cases, supporting the concept that chronic inflammatory signaling is closely intertwined with oral carcinogenesis. IL-8 contributes to leukocyte recruitment, angiogenesis, and local microenvironmental changes that may facilitate dysplastic persistence and progression. Similarly, the increased MMP-9 levels observed among cases are compatible with enhanced extracellular matrix turnover and basement membrane remodeling, processes that are central to tissue invasion and malignant transition. The positive correlations between miR-21 and IL-8 and between miR-31 and MMP-9 suggest that the measured microRNAs and proteins are not independent molecular events but may represent interrelated components of the same pathogenic network. This biological coherence strengthens the plausibility of the panel and supports its use as an integrated molecular signature rather than a collection of isolated biomarkers (13,14,18).

The diagnostic performance profile observed in this study is also notable in the context of existing literature. Previous salivary biomarker studies in oral precancer and oral squamous cell carcinoma have frequently reported encouraging results for individual miRNAs or cytokines, but direct comparison has been limited by variability in sample type, laboratory platform, normalization strategy, case definition, and lesion spectrum. The present results are aligned with earlier reports identifying miR-21, miR-31, and miR-184 as diagnostically relevant salivary biomarkers, while also extending that body of evidence by combining them with IL-8 and MMP-9 in one model. The superior AUC of the combined panel over the individual markers supports the view that multiplex approaches may be better suited to OPMDs, which are biologically heterogeneous and unlikely to be fully characterized by a single molecular signal. In practical terms, such integration may enhance triage performance by reducing the risk of under-recognizing lesions that express only part of the malignant phenotype (4,5,11,15).

From a clinical perspective, these findings support the potential role of saliva as a non-invasive adjunct in the early assessment of oral precancer. Saliva collection is simple, repeatable, and patient-friendly, which makes it suitable not only for specialist clinics but also for screening and surveillance in community settings. In high-risk populations with prevalent tobacco and betel nut exposure, a salivary biomarker panel could help identify patients requiring biopsy prioritization, shorter follow-up intervals, or referral to specialized care. Such a strategy would not replace histopathology, which remains essential

for definitive diagnosis, but it could complement conventional examination by enriching risk stratification before invasive tissue sampling. This is particularly relevant where patient reluctance, resource constraints, or delayed presentation hinder timely biopsy-based assessment (1,3,8,10).

Several methodological strengths support the internal consistency of the present findings. The study used standardized pre-analytical saliva collection, duplicate laboratory assays, blinded biomarker assessment, and uniform application of eligibility criteria. The inclusion of both microRNA and protein biomarkers allowed evaluation of distinct but biologically connected dimensions of oral carcinogenesis. In addition, the presentation of effect sizes and confidence intervals alongside p-values provides a clearer picture of the magnitude and precision of the observed differences, addressing a common limitation in biomarker reporting. The case group also included more than one OPMD subtype, allowing at least a preliminary appreciation of molecular variation across lesions with different clinical phenotypes (7,12,14).

Notwithstanding these strengths, the study has important limitations that must temper interpretation. First, the sample size was modest and the study was conducted at a single center over a relatively short period, which limits generalizability and reduces power for detailed subgroup analyses. Second, the cross-sectional design does not permit determination of whether the measured biomarkers can predict future malignant transformation, which is the most clinically meaningful endpoint in OPMD surveillance. Third, the marked imbalance in tobacco and betel nut exposure between cases and controls raises the possibility of residual confounding, since these behaviors may independently influence salivary inflammatory and molecular profiles. Although the observed biomarker differences were large, future studies should incorporate multivariable modeling or matched recruitment strategies to more rigorously separate lesion-related effects from exposure-related effects. Fourth, the study relied on single time-point saliva collection, which precludes assessment of intra-individual variability, diurnal fluctuation, and longitudinal stability. Finally, the absence of an independent external validation cohort means that the reported diagnostic accuracy should be considered promising but preliminary rather than definitive (16,17,19).

Future research should therefore move beyond cross-sectional discrimination and toward prospective validation. Larger multicenter studies are needed to confirm reproducibility across diverse populations, lesion sites, and exposure profiles. Longitudinal follow-up of OPMD patients would allow evaluation of whether these biomarkers predict dysplastic progression or malignant transformation over time, which would substantially strengthen their translational value. Further refinement of the panel may also be achieved by integrating additional analytes such as exosomal microRNAs, metabolomic markers, or other cytokines implicated in the oral premalignant microenvironment. In parallel, the application of multivariable prediction modeling and machine learning may help define optimal biomarker combinations, threshold values, and clinical decision rules for use in real-world screening pathways. Such developments could convert promising molecular signals into robust, clinically deployable tools for personalized oral cancer prevention (5,13,18,20).

Overall, the present study adds to the growing evidence that salivary molecular diagnostics can contribute meaningfully to the early detection of oral precancerous disease. The combined elevation of oncogenic microRNAs and protein mediators observed here reflects a biologically credible convergence of transcriptional dysregulation, inflammation, and matrix remodeling in high-risk OPMDs. Although further validation is essential, the findings indicate that a combined salivary biomarker panel has greater diagnostic utility than individual markers alone and may offer a feasible adjunctive strategy for early risk assessment in populations with substantial oral carcinogen exposure. By improving identification of lesions warranting closer evaluation, such approaches could support earlier intervention and potentially reduce progression to invasive oral cancer (8,10,14).

CONCLUSION

In conclusion, the present study found that salivary miR-21, miR-31, miR-184, IL-8, and MMP-9 were significantly elevated in patients with oral potentially malignant disorders and that their combined assessment provided stronger diagnostic discrimination than individual biomarkers alone. These findings support the potential role of saliva as a practical, non-invasive adjunct for early molecular risk assessment in OPMDs, particularly in high-risk populations with prevalent exposure to oral carcinogens. However, the results should be interpreted as preliminary diagnostic evidence rather than definitive validation, as larger prospective multicenter studies with adjustment for behavioral confounders and longitudinal follow-up are still needed before routine clinical implementation can be recommended.

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