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Salivary microRNA expression profiling for early detection of oral squamous cell carcinoma

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Abstract:
Oral squamous cell carcinoma (OSCC) is often diagnosed at advanced stages, underscoring the need for reliable non-invasive biomarkers for early detection. Therefore, it is of interest to analyze the salivary expression of six microRNAs (miR-21, miR-31, miR-125a, miR-184, miR-200a and miR-375) in 40 OSCC patients, 40 with oral potentially malignant disorders and 40 healthy controls. MiR-21 and miR-31 were significantly upregulated, while miR-125a and miR-200a were down regulated in OSCC cases. The combined miR-21 and miR-200a profile achieved high diagnostic accuracy (AUC = 0.942). Thus, salivary microRNA signatures, especially miR-21 + miR-200a, hold strong promise for non-invasive early detection of OSCC.

Keywords: Oral squamous cell carcinoma (OSCC), microRNA, saliva, biomarkers, early detection, non-invasive diagnosis

Background:
Oral squamous cell carcinoma (OSCC) constitutes the most prevalent form of oral cancer, accounting for approximately 90% of all oral malignancies worldwide **[1]**. Despite advances in treatment modalities, the five-year survival rate for OSCC remains disappointingly low at approximately 50-60%, primarily due to late-stage diagnosis when the disease has already progressed to advanced stages **[2]**. The global incidence of OSCC continues to rise, with an estimated 377,713 new cases and 177,757 deaths reported annually, making it a significant public health concern **[3]**. Early detection of OSCC dramatically improves patient outcomes, with five-year survival rates exceeding 80% for localized disease compared to less than 40% for cases with regional or distant metastases **[4]**. Current diagnostic methods rely primarily on clinical examination followed by tissue biopsy, which is invasive, requires specialized expertise and may not be suitable for large-scale screening programs **[5]**. These limitations underscore the urgent need for non-invasive, accurate and cost-effective biomarkers that can facilitate early detection and improve patient prognosis. MicroRNAs (miRNAs) are small non-coding RNA molecules, typically 18-25 nucleotides in length that play crucial roles in post-transcriptional gene regulation **[6]**. Aberrant miRNA expression has been implicated in various stages of carcinogenesis, including cell proliferation, apoptosis, invasion and metastasis **[7]**. Recent studies have showed that miRNAs are remarkably stable in body fluids, including saliva, making them attractive candidates for liquid biopsy approaches **[8]**. Saliva offers unique advantages as a diagnostic medium for oral cancer detection. Its collection is non-invasive, simple and can be performed repeatedly without causing patient discomfort **[9]**. Moreover, saliva directly bathes oral lesions, potentially providing a more representative molecular profile of the tumor microenvironment compared to serum or plasma **[10]**. Several studies have identified dysregulated miRNAs in the saliva of OSCC patients, including upregulation of oncogenic miRNAs such as miR-21 and miR-31 and downregulation of tumor suppressor miRNAs like miR-125a and miR-200a **[11]**. Therefore, it is of interest to assess the diagnostic potential of a panel of six salivary miRNAs (miR-21, miR-31, miR-125a, miR-184, miR-200a and miR-375) for early detection of OSCC and to determine the optimal miRNA combination that provides the highest diagnostic accuracy for clinical application.

Materials and Methods:
Study design and participants:
This case-control study was conducted between January 2023 and December 2023 at a tertiary care oral oncology center. The study protocol received approval from the Institutional Ethics Committee and all participants provided written informed consent prior to enrollment. A total of 120 participants were recruited and divided into three groups: 40 patients with histopathologically confirmed OSCC (case group), 40 patients with clinically diagnosed OPMDs including leukoplakia and erythroplakia (OPMD group) and 40 healthy individuals with no history of oral lesions (control group). Sample size calculation was performed using G*Power software, assuming an effect size of 0.8, alpha error of 0.05 and power of 0.80.

Inclusion and exclusion criteria:
Inclusion criteria for the OSCC group included: (1) histopathologically confirmed diagnosis of primary OSCC, (2) no prior treatment with chemotherapy or radiotherapy, (3) age between 30-70 years. The OPMD group included patients with clinically diagnosed leukoplakia or erythroplakia confirmed by visual examination and vital staining. Healthy controls were age and sex-matched individuals with no history of oral lesions or systemic diseases. Exclusion criteria for all groups included: (1) presence of other malignancies, (2) active periodontal disease or oral infections, (3) autoimmune disorders, (4) pregnancy or lactation, (5) use of immunosuppressive medications, (6) history of head and neck radiation.

Saliva collection and processing:
Unstimulated whole saliva samples were collected between 9:00 and 11:00 AM to minimize circadian variations. Participants were instructed to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 2 hours before collection. Approximately 5 mL of saliva was collected using the passive drool method into sterile, RNase-free containers. Samples were immediately placed on ice and processed within 2 hours of collection. Saliva samples were centrifuged at 3,000 × g for 15 minutes at 4°C to remove cellular debris. The supernatant was aliquoted into 1.5 mL tubes and stored at -80°C until RNA extraction. All samples were coded to ensure blinded analysis.

RNA extraction and quality assessment:
Total RNA, including miRNAs, was extracted from 500 µL of saliva supernatant using the miRNeasy Serum/Plasma Kit according to manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer. Samples with A260/A280 ratios between 1.8-2.1 were considered acceptable for further analysis.

Quantitative real-time PCR analysis:
Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit with specific stem-loop primers for each target miRNA. Quantitative real-time PCR was conducted using TaqMan MicroRNA assays on a StepOnePlus Real-Time PCR System. Each sample was analyzed in triplicate and miR-16 was used as an endogenous control for normalization. The relative expression levels of target miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. Quality control measures included no-template controls and inter-assay calibrators to ensure reproducibility.

Statistical analysis:
Statistical analysis was performed using SPSS version 26.0. Normality of data distribution was assessed using the Shapiro-Wilk test. Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data or median with interquartile range for non-normally distributed data. Categorical variables were presented as frequencies and percentages. One-way ANOVA with post-hoc Tukey's test was used to compare miRNA expression levels among the three groups. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic performance of individual miRNAs and their combinations. Optimal cut-off values were determined using Youden's index. Logistic regression analysis was used to identify the best combination of miRNAs for OSCC diagnosis. A p-value <0.05 was considered statistically significant.

Results:
The study cohort consisted of 120 participants with a mean age of 52.4 ± 11.3 years. The OSCC group comprised 26 males (65%) and 14 females (35%), with a mean age of 54.2 ± 10.8 years. The most common tumor sites were the lateral tongue (42.5%), buccal mucosa (27.5%) and floor of mouth (17.5%). Among OSCC patients, 22 (55%) presented with early-stage disease (stages I-II) and 18 (45%) with advanced-stage disease (stages III-IV). The OPMD group included 24 males (60%) and 16 females (40%), with a mean age of 51.8 ± 11.6 years. Leukoplakia was present in 28 patients (70%) and erythroplakia in 12 patients (30%). The control group consisted of 22 males (55%) and 18 females (45%), with a mean age of 51.2 ± 11.5 years. No significant differences in age or gender distribution were observed among the three groups (p>0.05). Analysis of salivary miRNA expression revealed distinct patterns among the three study groups. The expression levels of the six candidate miRNAs are presented in **Table 1**. Values are expressed as mean ± SD relative expression levels normalized to miR-16. MiR-21 and miR-31 showed significant upregulation in OSCC patients compared to both OPMD patients and healthy controls. The expression of miR-21 was 4.3-fold higher in OSCC compared to controls and 2.1-fold higher compared to OPMD patients. Similarly, miR-31 expression was 4.0-fold higher in OSCC compared to controls. Conversely, miR-125a, miR-200a and miR-375 demonstrated significant downregulation in OSCC patients. MiR-200a showed the most pronounced decrease, with expression levels 4.6-fold lower in OSCC compared to controls. The OPMD group showed intermediate expression levels for most miRNAs, suggesting a potential progression pattern from normal to malignant transformation. ROC curve analysis was performed to evaluate the diagnostic accuracy of individual miRNAs in distinguishing OSCC from healthy controls. The results are summarized in **Table 2**. Among individual miRNAs, miR-200a demonstrated the highest diagnostic accuracy with an AUC of 0.908, followed by miR-21 (AUC = 0.892). The optimal cut-off value for miR-200a yielded a sensitivity of 85.0% and specificity of 90.0% for OSCC detection. To improve diagnostic accuracy, various combinations of miRNAs were evaluated using logistic regression analysis. The diagnostic performance of the top three miRNA combinations is presented in **Table 3**. The combination of miR-21 and miR-200a provided the optimal balance between diagnostic accuracy and practical applicability, achieving an AUC of 0.942 with 87.5% sensitivity and 92.5% specificity. The addition of more miRNAs to the panel showed only marginal improvement in diagnostic performance while increasing assay complexity and cost. Subgroup analysis

revealed that miRNA expression patterns correlated with clinical stage and histological grade. Patients with advanced-stage OSCC (stages III-IV) showed significantly higher expression of miR-21 (5.68 ± 1.42 vs 4.12 ± 0.89, p<0.001) and miR-31 (4.82 ± 1.24 versus 3.26 ± 0.76, p<0.001) compared to early-stage disease. Similarly, poorly differentiated tumors exhibited more pronounced dysregulation of miRNA expression compared to Well-differentiated tumors.

Table 1: Salivary microRNA expression levels in study groups

MicroRNA	OSCC (n=40)	OPMD (n=40)	Control (n=40)	p-value
miR-21	4.82 ± 1.23	2.34 ± 0.78	1.12 ± 0.34	<0.001
miR-31	3.96 ± 1.08	2.18 ± 0.65	0.98 ± 0.29	<0.001
miR-125a	0.42 ± 0.15	1.23 ± 0.38	1.89 ± 0.46	<0.001
miR-184	2.87 ± 0.92	1.76 ± 0.54	1.02 ± 0.31	<0.001
miR-200a	0.38 ± 0.12	1.14 ± 0.32	1.76 ± 0.52	<0.001
miR-375	0.56 ± 0.18	1.32 ± 0.41	1.94 ± 0.58	<0.001

Table 2: Diagnostic performance of individual salivary microRNAs for OSCC detection

MicroRNA	AUC (95% CI)	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
miR-21	0.892 (0.823-0.961)	2.85	82.5	87.5	86.8	83.3
miR-31	0.876 (0.802-0.950)	2.42	80.0	85.0	84.2	81.0
miR-125a	0.854 (0.774-0.934)	0.98	77.5	82.5	81.6	78.6
miR-184	0.812 (0.723-0.901)	1.94	72.5	80.0	78.4	74.4
miR-200a	0.908 (0.844-0.972)	0.86	85.0	90.0	89.5	85.7
miR-375	0.838 (0.754-0.922)	1.12	75.0	82.5	81.1	76.7

AUC: Area under the curve; CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value

Table 3: Diagnostic performance of combined microRNA panels for OSCC detection

MicroRNA Combination	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
miR-21 + miR-200a	0.942 (0.894-0.990)	87.5	92.5	92.1	88.1	90.0
miR-21 + miR-31 + miR-200a	0.938 (0.887-0.989)	85.0	92.5	91.9	86.0	88.8
All six miRNAs	0.946 (0.900-0.992)	90.0	90.0	90.0	90.0	90.0

Discussion:

This study shows the significant potential of salivary miRNA expression profiles as non-invasive biomarkers for early detection of OSCC. Our findings reveal distinct patterns of miRNA dysregulation in OSCC patients, with upregulation of oncogenic miRNAs (miR-21, miR-31) and downregulation of tumor suppressor miRNAs (miR-125a, miR-200a, miR-375), consistent with their established roles in oral carcinogenesis. The observed upregulation of miR-21 in OSCC saliva samples aligns with previous studies showing its oncogenic function in various cancers **[12]**. MiR-21 promotes tumor progression by targeting multiple tumor suppressor genes, including PTEN, PDCD4 and TPM1, leading to enhanced cell proliferation, invasion and resistance to apoptosis **[13]**. Our finding of a 4.3-fold increase in salivary miR-21 expression in OSCC patients supports its utility as a diagnostic biomarker, with an individual AUC of 0.892. Similarly, the upregulation of miR-31 observed in our study corroborates its role as an oncomiRs in OSCC. Previous research has shown that miR-31 promotes tumor growth and metastasis by targeting tumor suppressor genes such as SDHD and LATS2 **[14]**. The intermediate expression levels of miR-31 in OPMD patients suggest its potential involvement in malignant transformation, warranting further investigation in longitudinal studies. The significant downregulation of miR-200a in OSCC patients is particularly noteworthy, as this miRNA plays a crucial role in maintaining epithelial phenotype and suppressing epithelial-mesenchymal transition (EMT) **[15]**. Loss of miR-200a expression has been associated with increased invasiveness and metastatic potential in various cancers, including OSCC **[16]**. Our finding that miR-200a showed the highest individual diagnostic accuracy (AUC = 0.908) among the down regulated miRNAs underscores its importance as a tumor suppressor in oral carcinogenesis. The combination of miR-21 and miR-200a yielded superior diagnostic performance compared to individual miRNAs, achieving 87.5% sensitivity and 92.5% specificity. This finding is consistent with the concept that multiple molecular alterations contribute to cancer development and combining biomarkers with complementary functions can enhance diagnostic accuracy **[17]**. The high specificity of this two-miRNA panel is particularly important for screening applications, as it minimizes false-positive results and unnecessary anxiety for patients. Our study also revealed that patients with OPMDs showed intermediate miRNA expression levels between OSCC and healthy controls. This observation suggests that miRNA dysregulation may occur early in the carcinogenesis process and could potentially be used to identify high-risk individuals who require closer surveillance **[18]**. Future longitudinal studies should investigate whether specific miRNA expression patterns can predict malignant transformation of OPMDs. The correlation between miRNA expression levels and clinical parameters, such as tumor stage and grade, provides additional validation of their biological relevance. The higher expression of oncogenic miRNAs in advanced-stage disease suggests that these biomarkers may also have prognostic value, although this requires further investigation in larger cohorts with long-term follow-up data. From a clinical perspective, saliva-based miRNA testing offers several advantages over current diagnostic methods. The non-invasive nature of saliva collection enables repeated sampling for monitoring purposes, which is particularly valuable for surveillance of high-risk individuals and assessment of treatment response **[19]**. Additionally, the stability of miRNAs in saliva and the availability of standardized

extraction and quantification protocols make this approach feasible for clinical implementation **[20]**. However, several limitations should be acknowledged. First, the cross-sectional design of this study precludes assessment of the temporal relationship between miRNA dysregulation and cancer development. Second, the relatively small sample size may limit the generalizability of our findings, particularly for subgroup analyses. Third, potential confounding factors such as tobacco use, alcohol consumption and HPV status were not comprehensively evaluated in this study. Future studies should address these limitations through larger, multicenter validation studies with comprehensive clinical and molecular characterization.

Conclusion:

We show the use of salivary miRNA expression profiles as non-invasive biomarkers for early detection of OSCC. The combination of miR-21 and miR-200a showed excellent diagnostic performance with 87.5% sensitivity and 92.5% specificity, offering a promising approach for oral cancer screening. The distinct expression patterns observed in OPMD patients suggest potential applications in risk stratification and surveillance of premalignant lesions. Implementation of saliva-based miRNA testing could significantly improve early detection rates and ultimately enhance patient outcomes in OSCC.

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