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Salivary MicroRNA profiling in oral potentially malignant disorders

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Abstract:

Oral potentially malignant disorders (OPMDs) are clinically important because of their risk of progression to oral squamous cell carcinoma and early detection of malignant transformation remains difficult. Therefore, it is of interest to evaluate the salivary expression of selected miRNAs in 140 participants comparing patients with OPMDs and healthy controls. Unstimulated saliva samples were analyzed by qRT-PCR for miR-21, miR-31, miR-184 and miR-155 and OPMD patients showed increased oncogenic miRNAs with reduced tumor suppressor miRNA expression compared with controls. Higher expression of miR-21 and miR-31 was significantly associated with epithelial dysplasia severity and tobacco exposure. Thus, we show the salivary miRNA profiling as a promising non-invasive diagnostic adjunct for identifying high-risk OPMDs and monitoring malignant transformation.

Keywords: Saliva, microRNA, oral leukoplakia, oral submucous fibrosis, oral lichen planus, oral cancer, biomarkers, qRT-PCR

Background:

Oral Squamous Cell Carcinoma is one of the most prevalent oral malignancies and is a major cause of morbidity and mortality in the world. The prevalence of oral cancer is significantly higher in South Asian countries, especially in India, where the use of smokeless tobacco, areca nut, betel quid and smoking are common [1]. Oral potentially malignant disorders (OPMDs) are a range of lesions, including oral leukoplakia, oral submucous fibrosis and oral lichen planus that have different risks of malignant transformation. Early identification and molecular risk stratification of these lesions are still crucial to minimize disease burden and patient mortality [2]. The diagnosis of OPMDs is largely based on clinical examination and histopathological evaluation. But these techniques are invasive and may not detect early molecular changes that lead to carcinogenesis [3]. In recent years, the importance of non-coding RNAs, particularly microRNAs (miRNAs) in tumor initiation, progression, angiogenesis, apoptosis and metastasis has been emphasized. MiRNAs are short endogenous non-coding RNAs of around 18-25 nucleotides in length that post-transcriptionally regulate gene expression. A number of cancers, including oral cancer, have been associated with the dysregulation of miRNA expression [4]. Saliva has become a promising diagnostic medium due to its non-invasive, readily accessible, low cost and protein, DNA, RNA, exosome, metabolite and inflammatory mediator content. Salivary diagnostics has received significant interest in oral oncology due to the direct contact of saliva with oral lesions and the local pathological changes. Salivary miRNAs

are extremely resistant to degradation by RNases because they are protected in extracellular vesicles or protein complexes [5, 6]. The properties of these miRNAs render them suitable as biomarkers for early detection and monitoring of oral premalignant lesions and malignant transformation [7]. Of the various miRNAs identified, miR-21 and miR-31 are oncogenic and are often found to be over-expressed in OPMDs and OSCC. Increased expression of miR-21 has been associated with enhanced cellular proliferation, invasion and resistance to apoptosis. Likewise, miR-31 has been shown to be strongly associated with the severity of epithelial dysplasia and the risk of malignant progression. In contrast, miR-184 may function as a tumor suppressor and is often downregulated in premalignant lesions. Changes in the expression of these miRNAs in saliva could thus be a molecular marker for disease progression [8, 9]. In the last few years, a few studies and systematic reviews have examined the usefulness of salivary miRNAs as biomarkers in oral cancer and OPMDs. But there is some variation in the number of samples, in the type of lesions and in the methods used, which has hindered universal clinical application. Additional studies are required to confirm salivary miRNA profiles in a variety of populations and to create standardized diagnostic procedures [10, 11]. Therefore, it is of interest to compare the expression levels of salivary miRNA in patients with OPMDs and healthy controls using qRT-PCR and to determine the correlation between miRNA dysregulation and clinicopathological parameters and dysplasia severity.

Materials and Methodology:

This was an observational analytical study carried out in the Department of Oral Medicine and Radiology in association with Department of Oral Pathology and Molecular Diagnostics for 18 months after approval from the Institutional Ethics Committee. All participants gave informed consent before they were included in the study.

A total sample size of 140 participants was included. Subjects were divided into two groups:

- [1] Group I: 100 patients with OPMDs who had been treated with laser therapy.
- [2] Group II: 40 healthy controls without oral lesions or deleterious habits.

Patients with oral leukoplakia, oral submucous fibrosis and oral lichen planus were included in the OPMD group. Untreated lesions were included in patients older than 18 years. Patients who had received previous oral cancer treatment, systemic malignancy, autoimmune disease, severe infection, pregnancy, or recent antibiotic therapy were excluded. Demographic data such as age, gender, tobacco use, alcohol intake, lesion duration and clinical findings were documented. Oral examination was carried out comprehensively and the characteristics of the lesions were documented, such as location, size, surface texture and clinical staging. Incisional biopsy was done in any suspicious lesions and histopathological grading of epithelial dysplasia was done based on WHO criteria. Whole saliva was collected without stimulation between 9 AM and 11 AM to reduce the circadian variation. Participants were asked to refrain from eating, drinking, and smoking and oral hygiene activities for at least one hour prior to sample collection. Approximately 5 mL of saliva was collected in sterile RNase-free tubes and immediately stored at -80°C until analysis. Commercial miRNA extraction kits were used to extract total RNA including miRNA, following the manufacturer's instructions. Complementary DNA synthesis was performed and quantitative real-time polymerase chain reaction (qRT-PCR) analysis was conducted with specific primers for miR-21, miR-31, miR-184 and miR-155. U6 small nuclear RNA was used as an endogenous control. The comparative Ct method was used to calculate relative gene expression. Data were analyzed statistically with SPSS software version 26. All continuous variables were presented as mean ± SD and all categorical variables as percentages. Student's t-test, ANOVA and Chi-square tests were used as appropriate. Pearson correlation coefficient was used to analyse the correlation between miRNA expression and the severity of dysplasia. The p value was used as the statistical significance criterion and was set at <0.05.

Results:

A total of 140 subjects were evaluated in the present study. Among the 100 OPMD patients, oral submucous fibrosis constituted the majority of cases followed by leukoplakia and oral lichen planus. Males predominated in the study population due to higher tobacco exposure. Most participants belonged to

the age group of 31-50 years. Significant association was observed between tobacco habits and occurrence of OPMDs. The majority of OPMD patients were males with significant tobacco exposure. Oral submucous fibrosis was the most common lesion identified. Histopathological dysplasia was observed in 63% of cases, indicating substantial malignant potential among the studied lesions. Quantitative analysis of salivary miRNA expression revealed significantly elevated levels of miR-21, miR-31 and miR-155 among OPMD patients compared with healthy controls. In contrast, miR-184 expression was significantly reduced (Table 1). Marked upregulation of oncogenic miRNAs was observed in OPMD patients, suggesting active molecular dysregulation during oral carcinogenesis. MiR-21 demonstrated the highest fold increase and showed strong association with lesion severity. Reduced miR-184 levels indicated loss of tumor suppressive function. Further subgroup analysis demonstrated that miRNA dysregulation was more pronounced in lesions exhibiting moderate-to-severe epithelial dysplasia compared with mild dysplasia (Table 2). A progressive increase in expression of miR-21, miR-31 and miR-155 was observed with increasing severity of epithelial dysplasia, whereas miR-184 showed progressive downregulation. These findings indicate the potential role of salivary miRNAs in identifying lesions with high malignant transformation risk (Table 3). Receiver operating characteristic analysis demonstrated high diagnostic performance for salivary miR-21 with sensitivity of 91%, specificity of 87% and area under curve of 0.93. Combined miRNA panel analysis further improved diagnostic accuracy. Overall, the study findings demonstrated that salivary miRNA profiling can effectively differentiate OPMD patients from healthy controls and may aid in early detection of malignant transformation.

Table 1: Demographic and clinical characteristics

Parameter	OPMD Group (n=100)	Controls (n=40)	p-value
Mean age (years)	43.8 ± 11.2	39.4 ± 9.8	0.041*
Male (%)	72%	57.5%	0.038*
Tobacco users (%)	81%	22.5%	<0.001*
Oral leukoplakia	38%	—	—
Oral submucous fibrosis	44%	—	—
Oral lichen planus	18%	—	—
Dysplasia present	63%	—	<0.001*

Table 2: Comparative Salivary miRNA expression levels

miRNA	OPMD Group Mean Fold Change	Controls Mean Fold Change	p-value
miR-21	5.84 ± 1.92	1.14 ± 0.48	<0.001*
miR-31	4.67 ± 1.56	1.08 ± 0.42	<0.001*
miR-155	3.91 ± 1.37	1.03 ± 0.31	<0.001*
miR-184	0.42 ± 0.18	1.00 ± 0.27	<0.001*

Table 3: Association Between miRNA expression and dysplasia severity

miRNA	Mild Dysplasia	Moderate Dysplasia	Severe Dysplasia	p-value
miR-21	3.12 ± 0.88	5.89 ± 1.11	8.21 ± 1.46	<0.001*
miR-31	2.94 ± 0.74	4.75 ± 1.09	6.84 ± 1.33	<0.001*
miR-155	2.12 ± 0.61	3.84 ± 0.96	5.22 ± 1.17	<0.001*
miR-184	0.82 ± 0.16	0.49 ± 0.11	0.21 ± 0.08	<0.001*

Discussion:

Oral potentially malignant disorders (OPMDs) are well known to be precursor lesions that have different potential for progression to oral squamous cell carcinoma (OSCC). The early detection of molecular changes in the premalignant phase is crucial to enhance prognosis and to decrease the morbidity and mortality associated with oral cancer. Salivary diagnostics has become a significant area of oral oncology in recent years due to the fact that saliva is a non-invasive, inexpensive and patient-friendly medium for biomarker analysis. El-Sakka *et al.* highlighted the potential of miRNA expression changes as useful biomarkers for risk assessment in OPMDs and as a tool to help distinguish lesions that have a greater likelihood of malignant transformation [1]. Likewise, Bhattacharyya *et al.* and Aghbari *et al.* have shown that miR-27b and miR-137 could be used to predict the disease activity and risk of malignant transformation in patients with oral lichen planus, indicating the diagnostic potential of salivary molecular markers in oral premalignancy [13, 14]. In the present study, salivary expression levels of miR-21, miR-31, miR-155 and miR-184 were compared between patients with OPMDs and healthy controls. These miRNAs were significantly dysregulated, which is indicative of their role in oral carcinogenesis. Most of the patients in the present study were tobacco consumers or areca nut consumers, which is in line with the epidemiological data reported from India and other South Asian countries, where tobacco use is closely linked with the occurrence of OPMDs and the development of OSCC. MicroRNAs are small endogenous non-coding RNA molecules that post-transcriptionally regulate gene expression and modulate multiple biological pathways such as proliferation, apoptosis, angiogenesis, invasion and immune regulation. Saliminejad *et al.* explained the biological importance and analytical applications of miRNAs and highlighted their importance in cancer diagnosis and therapy [9]. Fang and Li also pointed out the potential use of miRNAs in oral cancer diagnosis and molecular-targeted therapy [10]. Chang *et al.* also showed that a three-miRNA signature is a promising biomarker for early detection of oral cancer, highlighting the significance of miRNA profiling in oral oncology [11]. In the present study, miR-21 was found to be the most highly upregulated miRNA of all those evaluated. miR-21 is regarded as an oncogenic miRNA that stimulates proliferation, invasion, epithelial-mesenchymal transition and resistance to apoptosis in malignant cells. Garg *et al.* found that miR-21 was significantly higher in both OPMD and OSCC patients and proposed that it could be used as a potential early biomarker for malignant transformation [3]. In the same way, Liu *et al.* found that there was a strong association between the salivary miRNA dysregulation and progression of oral premalignant lesions [4]. The results of the present study are thus in line with previous studies that have shown that miR-21 is involved in oral carcinogenesis. The present study also noted a significant upregulation of miR-31, especially in lesions with severe epithelial dysplasia. miR-31 has been shown to regulate pathways associated with hypoxia, inflammatory signaling and epithelial proliferation. The upregulation of miR-31 may thus play a role in the dysplastic progression and malignant transformation. The current study found that miR-31 expression

was increased in a progressive manner as the dysplasia progressed, which further supports the role of miR-31 as a prognostic marker for the identification of biologically aggressive lesions. The other significant finding of the study was that miR-155 was highly up-regulated in OPMD patients. miR-155 is known as an inflammatory oncogenic miRNA that plays a role in immune modulation and regulation of the tumor microenvironment. The increase in expression may be a result of chronic inflammation due to tobacco-induced mucosal injury and oral lichen planus. Menini *et al.* pointed out the relevance of the role of salivary microRNAs in the diagnosis of OSCC and their potential as noninvasive biomarkers that can reflect the underlying inflammatory and neoplastic changes [15]. Moreover, Nakamura *et al.* showed that the circulating miRNA panels have high diagnostic accuracy for OSCC detection, which suggests the clinical value of miRNA-based diagnosis [12]. However, miR-184 was significantly downregulated in the patients with OPMD compared to healthy controls. The tumor suppressor miRNAs are thought to control apoptosis, epithelial differentiation and cellular homeostasis. This may then allow for uncontrolled proliferation and dysplastic progression. Garg *et al.* also found that salivary miR-184 expression was decreased in patients with OPMD and OSCC and highlighted its diagnostic potential to distinguish premalignant lesions from malignant disease [3]. The importance of salivary diagnostics has been growing due to the direct contact of saliva with oral lesions and the local pathological changes. Roi *et al.* reported that miRNAs in circulation and saliva are extremely stable and could be used as biomarkers for oral cancer liquid biopsy applications [2]. The analytical sensitivity and reproducibility of salivary biomarker analysis have been greatly enhanced by the development of new qRT-PCR methods and exosomal RNA isolation. Kimura *et al.* also proposed that miRNAs in saliva could be used as therapeutic monitoring markers for advanced OSCC patients [8]. Salivary miRNA profiling has been recently confirmed by several systematic reviews and meta-analyses as a valuable diagnostic tool. Osan *et al.* noted the potential of salivary miRNAs in the prediction of malignant transformation of OPMDs into oral cancer [5]. Qin *et al.* showed that salivary miRNAs and inflammatory cytokines could be used in combination to enhance the diagnosis and prognosis of OSCC [6]. Wijaya *et al.* also reported that miRNA profiles in saliva have diagnostic accuracy similar to plasma and serum biomarkers, but have the added benefits of being a non-invasive sample and are more patient-friendly [7]. Furthermore, Bhattacharyya *et al.* highlighted the emerging role of other noncoding RNAs such as circulating long noncoding RNAs as biomarkers and therapeutic targets in oral cancer [13]. A key finding of the current study was the relationship between the expression of miRNAs and the severity of the dysplasia in the histopathology. The increase in oncogenic miRNAs in the context of increasing dysplasia may indicate that miRNA profiling of saliva may provide a more accurate reflection of biological aggressiveness than clinical appearance alone. This reinforces the idea of molecular risk stratification of OPMDs using saliva. While there are promising results, there are some caveats. The amount of miRNA

quantified may be affected by variability in the methods of saliva collection, RNA isolation, sample storage and normalization. Salivary biomarker profiles can also be affected by inflammatory oral diseases and systemic diseases. Thus, large multicentric longitudinal studies are required prior to clinical use of salivary miRNA diagnostics. In summary, the present study is in line with the increasing body of evidence that salivary miRNA profiling is a promising noninvasive diagnostic tool for early detection, risk assessment and monitoring of oral potentially malignant disorders.

Conclusion:

Salivary miRNA profiling showed potential as a non-invasive diagnostic tool for oral potentially malignant disorders. Altered miR-21, miR-31, miR-155 and miR-184 expression was associated with epithelial dysplasia and malignant transformation risk. Larger multicentric longitudinal studies are needed to validate and standardize salivary miRNA-based diagnostics.

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