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Original Article

Study to explore the significance of saliva as a diagnostic tool to detect microRNA in oral potentially malignant disorders

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ABSTRACT

Oral carcinogenesis is a complex multistep process and there is a need for discovery of novel biomarkers such as salivary microRNA (miRNA) for early diagnosis as 16–62% of OSCC develops from oral potentially malignant disorders (OPMDs). Oral tissues are immersed in saliva; hence, saliva is a non-invasive reliable indicator for early malignant changes in oral epithelial precursor lesions. The role of miRNA as signatures of carcinogenesis though well established *in vitro* and *in vivo*, there are no studies evaluating the expression of salivary miRNA in Indian population. This study is the forerunner to study more genomic novel markers in saliva proving the diagnostic potential of saliva miRNA in early diagnosis of malignancy. The study mainly aims in evaluating whether saliva being a non-invasive diagnostic fluid can be used as a reliable source to study miRNA expression mainly miRNA 21 and miRNA 31 as a biomarker to detect early dysplastic changes in OPMDs. The study had proved that miRNA 21 and 31 are definitely expressed in both control and OPMDs in saliva, and there is significant downregulation of gene expression seen in OPMDs with average fold expression of miRNA 21 calculated as 58.48 ($P = 0.016$) and average fold expression of miRNA 31 as 67.18 ($P = 0.014$).

Keywords: Early dysplasia, microRNA 21, microRNA 31, novel genomic marker, salivary microRNA

Introduction

Clinicians have reported that the main problem encountered in oral cancer is the late diagnosis leading to lymphatic spread and recurrence. Recent literature has reported that clinical and pathological variables cannot assess early malignant changes in oral precursor lesions.[1] There are thousands of genes transcribing messenger RNA, microRNA (miRNA), etc. Hence, cancer development is multistep complex process demanding the need for identification of novel biomarkers such as miRNA which have been proved in recent studies that they are the signatures of oral carcinogenesis.[2] Oral tissues are immersed in saliva; hence, recent studies have proved that saliva is not just a surrogate marker but has proved to be more significant in expressing biomarker.[3,4] Salivary miRNA expression studies will be helpful in early diagnosis and prevention of malignant transformation of oral potentially malignant disorders (OPMDs).[5] Null hypothesis of the current research suggests that there is no significant difference in expression of salivary miRNA 21 and 31 between control and OPMDs and alternate hypothesis suggests significant difference in expression of salivary miRNA 21 and 31 between control and OPMDs.

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Aim

The present diagnostic study aims at evaluating the expression of salivary miRNA 21and 31 in OPMDs.

Objectives

1. To evaluate whether miRNA is expressed in saliva.
2. To study the expression of salivary miRNA 21 and 31 in varying dysplastic OPMDs.
3. To evaluate whether there is significant difference in expression of salivary miRNA between healthy and varying degree of dysplastic lesions

Materials and Methods

Materials

Sample size was calculated using G Power software using single mean sample size determination and was calculated as 18 per group for power of 80%. Judgement sampling as OPMDs was examined clinically initially and then included in the study. Initially, a pilot study after ethical clearance [032/02/2017/IEC/SU] was started with 10 samples including five control and five test samples to evaluate whether miRNA expression occurs in saliva. Inclusion criteria include OPMDs, namely, oral leukoplakia, oral lichen planus, and oral submucous fibrosis. Exclusion criteria include patients who are undergoing treatment for OPMDs.

Methods

Sample collection

Unstimulated whole saliva from the participants was collected between 6 and 11 am after providing "patient information sheet" and obtaining informed consent duly signed by the participant. Participants were asked to refrain from eating, drinking, or applying oral hygiene procedures on the day of saliva collection. Participants were instructed to mouth rinse with water before collection to avoid contamination. Saliva samples were collected using spitting method and 8–10 ml was collected in 50 ml falcon tube kept on dry ice.

Saliva processing

Saliva centrifugation at 2600 g 15 min 4°C was done to obtain supernatant saliva followed by RNA extraction done using RNase inhibitor. Samples were aliquoted in 440 µl and stored at –80°C until further use. Salivary total RNA isolation by RNeasy kit (Qiagen) according to the manufacturer's protocol from the same amount of collected saliva for each subject was done using Nanodrop spectrophotometer for quantification of the extracted RNA. Taqman miRNA Reverse Transcription in master mix was prepared in polypropylene tube by scaling the volumes to the desired number of RT reactions. Centrifugation and the RT master mix were placed on ice until miRNA reaction and non-template control in reaction was used in the reaction setup to avoid contamination during reaction and also to confirm the non-specific amplification for study of miRNA expression, namely, hsa-miR-21-5p MIMAT0000076 UAGCUUAUCAGACUGAUGUUGA, hsa-miR-31-5p (AGGCAAGAUGCUGGCAUAGCU), and hsa-miR-16-5p MIMAT0000069 UAGCAGCACGUAAAUAUUGGCG. MiRNA 16-5p was used as reference gene and miRNA 21-5p and miRNA 31-5p were the target genes.[6]

Results

The diagnostic study has proved the significant difference with down-regulated expression of salivary miRNA in all OPMDs (PMD) with mild dysplasia cases when compared to healthy control samples. Clinically diagnosed and histopathologically investigated cases were alone included in the study [Figures 1-5]. The demographic data, adverse habits, and histopathological features of the test samples

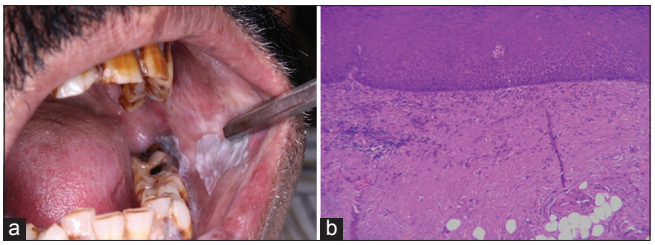


Figure 1: (a) Oral submucous fibrosis with leukoplakia, (b) hyperkeratosis with mild dysplasia

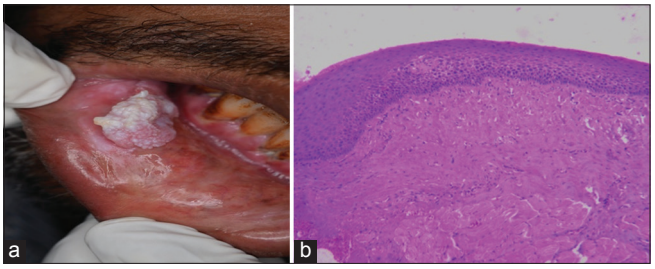


Figure 2: (a) Verrucous leukoplakia, (b) verrucous hyperplasia

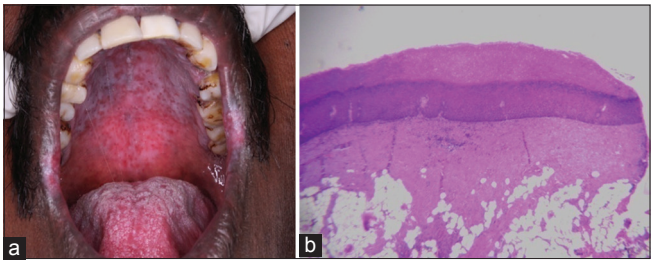


Figure 3: (a) Candidal leukoplakia, (b) hyperkeratosis with mild dysplasia

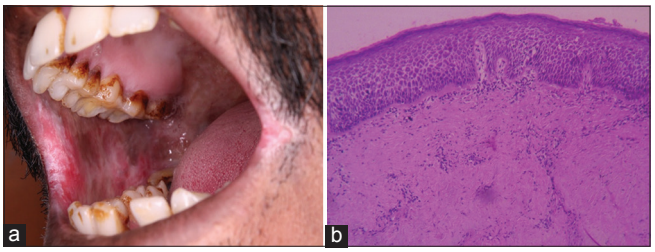


Figure 4: (a) Oral submucous fibrosis (OSMF), (b) advanced OSMF with mild dysplasia with leukoplakia

are tabulated [Table 1]. The CT value or cycle threshold defines the number of cycles required for the fluorescent signal to cross the threshold.[7] The average CT values of salivary miRNA 21 and 31 in control versus OPMDs were calculated for all the samples [Tables 2 and 3] and delta CT values were calculated by evaluating the difference in CT values of target genes in each of the samples with that of the reference gene and compared between control and test samples [Graphs 1 and 2]. The fold expression reveals that salivary miRNA is significantly downregulated in test samples when compared to control with $P = 0.016$ for miRNA 21 and $P = 0.016$ for miRNA 31 proving the significance of study of salivary miRNA in diagnosing early dysplastic changes in OPMDs [Table 4] average CT values of miRNA 21 (36.84) when compared to miRNA 31 (39.99) in test samples

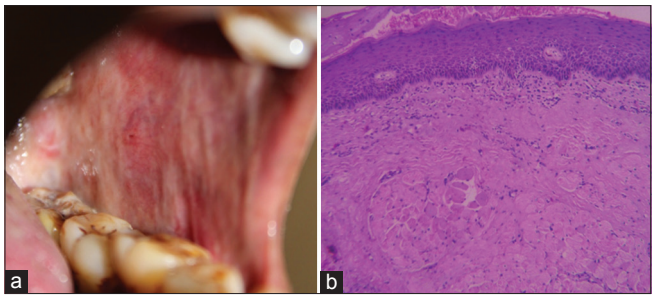


Figure 5: (a) Oral submucous fibrosis (OSMF), (b) OSMF with mild epithelial dysplasia

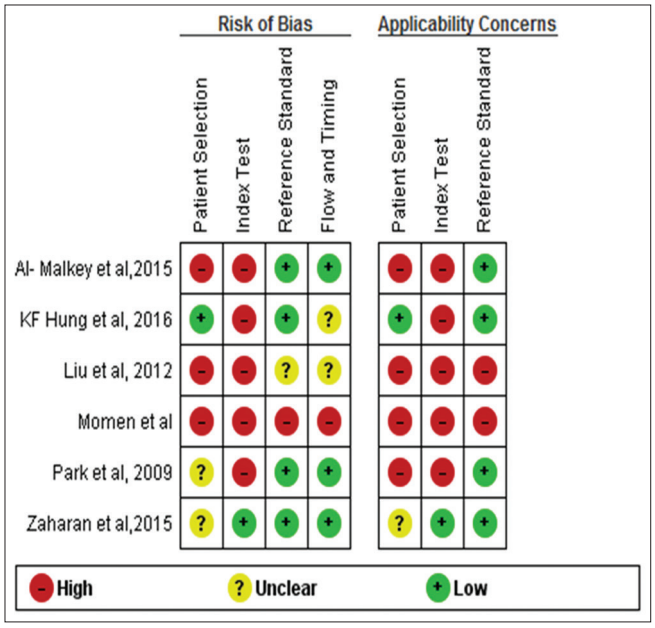
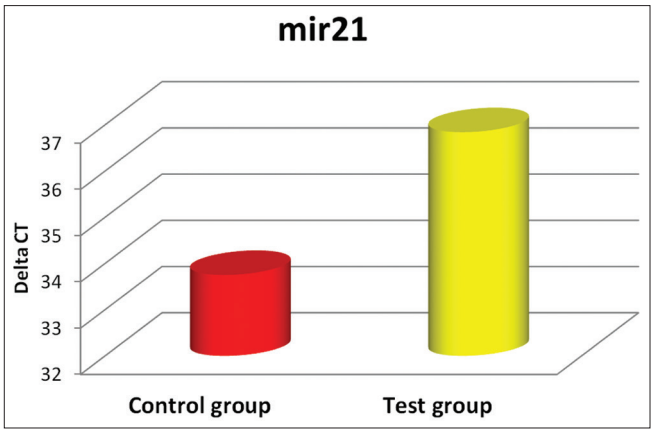


Figure 6: Risk of bias chart of six studies included in systematic review done using RevMan 5.3

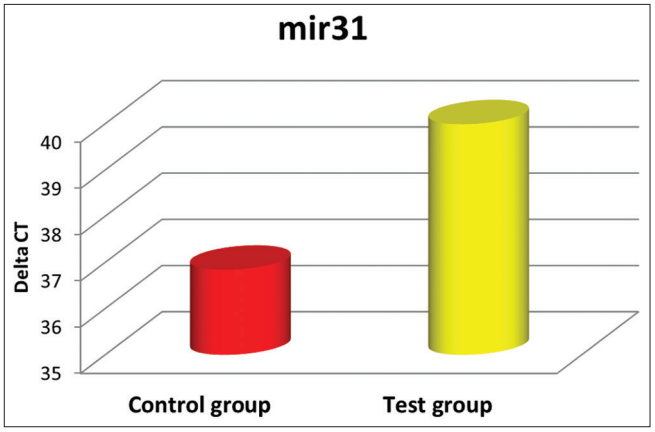
proves that expression of salivary miRNA 21 is more significant as lesser the CT values higher the expression [Graphs 3 and 4].

Discussion

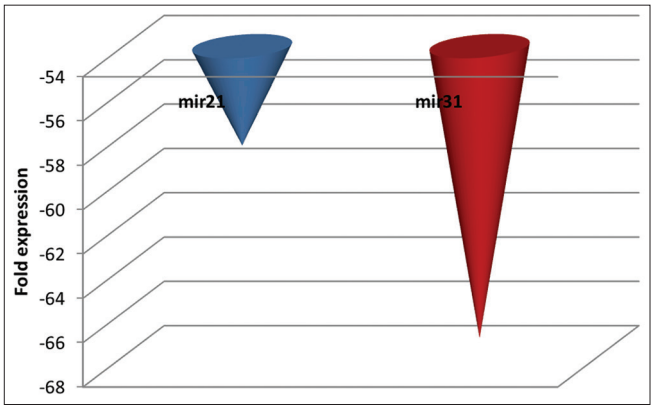
MiRNA in saliva as a viable marker for oral carcinoma was first developed in 2009.[8] A systematic review was first done before starting the pilot study to evaluate the significance of salivary miRNA in OPMDs. Around 574 studies were identified from web search, from which after applying human filter 197 studies were obtained. 2 studies were identified from manual searching, so a total of 199 studies were retrieved. 175 studies were excluded after applying the inclusion (studies done in salivary microRNA of OPMDs and oral cancer) and exclusion criteria (studies done in miRNA of serum, plasma or tissues of both OPMDs, and oral cancers). A total of six studies, that were done in the saliva samples of OPMDs and oral cancer were included in the systematic review.[6] Two studies Momen *et al.* and Zahran *et al.* have proved with statistical evidence of sensitivity and specificity for four salivary miRNAs, namely, miRNA 27b, miRNA 145, miRNA 181, and miRNA 21.[9,10] There is only one study that has compared the saliva samples with the tissue samples and has



Graph 1: Delta CT values of salivary microRNA 21 in control and oral potentially malignant disorders



Graph 2: Delta CT values of salivary microRNA 31 in control and oral potentially malignant disorders



Graph 3: Comparison of fold expression of salivary microRNA 21 and 31

concluded that saliva samples are significant predictors than tissue and miRNA 31 is a significant marker than miRNA 21 in saliva samples of OPMDs.[11] The systematic review concluded that five miRNAs, namely, miRNA-31, miRNA-24, miRNA-27b, miRNA-21, miRNA-184 are upregulated and 15 miRNAs, namely, miRNA-200a, miRNA-125a, miRNA-11, miRNA-191, miRNA-136, miRNA-147, miRNA-1250, miRNA-632, miRNA-646, miRNA-668, miRNA-877, miRNA-503, miRNA-200a, miRNA-323-5, and miRNA-145 are downregulated

Table 1: Demographic, tobacco history, and histopathological data of PMD cases

Code no	Age/sex	Tobacco history	Diagnosis	Histopathology report
T1	56/M	Slaked lime and betel nut 4 times/day for 30 years	Oral submucous fibrosis in the right and left buccal mucosa, homogeneous oral leukoplakia in the left buccal mucosa	Hyperorthokeratinized stratified epithelium of variable thickness with mild epithelial dysplasia and advanced oral submucous fibrosis
T2	33/M	Betel nut 8–10 packets/day for 5 years	Verrucous leukoplakia	Hyperparakeratosis with sharp rete pegs parakeratin plugging with epithelial hyperplasia suggestive of verrucous hyperplasia
T3	36/M	Beedi smoking 15/day for 10 years	Candidal leukoplakia in the left and right buccal mucosa	Hyperkeratosis with mild epithelial dysplasia
T4	37/M	Mawa chewing 5 packets/day for 16 years	Oral submucous fibrosis in the right and left buccal mucosa, oral leukoplakia in the right and left buccal mucosa	Parakeratinized stratified squamous epithelium with atrophic and loss of rete pegs bilaterally suggestive of advanced oral submucous fibrosis with mild epithelial dysplasia
T5	64/M	Pan chewing 3-4 packets/14 years	Oral submucous fibrosis in the left buccal mucosa	Oral submucous fibrosis with mild epithelial dysplasia

Table 2: CT and delta CT values of salivary miRNA 21 in control and OPMDs (PMD)

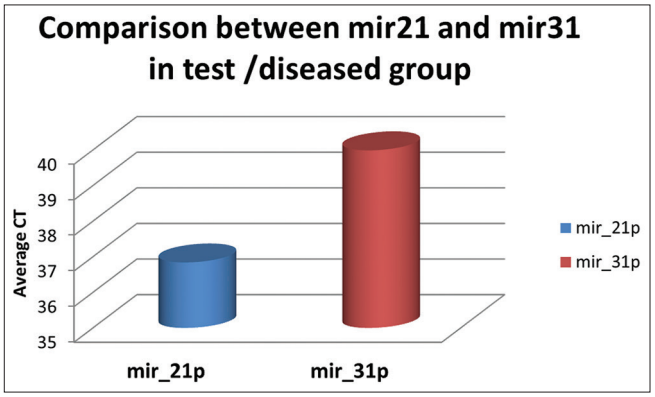
Sample	Gene	CT	Sample	Gene	CT	Delta CT
CON1	mir_16p	32.75	CON1	mir_21p	37.10	4.34
CON2	mir_16p	32.80	CON2	mir_21p	35.26	2.46
CON3	mir_16p	33.54	CON3	mir_21p	32.19	1.35
CON4	mir_16p	30.40	CON4	mir_21p	33.34	2.94
CON5	mir_16p	35.78	CON5	mir_21p	30.85	4.93
				Average	33.75	0.69
T1	mir_16p	30.89	T1	mir_21p	41.18	10.29
T2	mir_16p	30.25	T2	mir_21p	34.77	4.52
T3	mir_16p	29.69	T3	mir_21p	36.52	6.82
T4	mir_16p	29.30	T4	mir_21p	33.27	3.97
T5	mir_16p	31.24	T5	mir_21p	38.45	7.21
				Average	36.84	6.56

OPMDs: Oral potentially malignant disorders, MiRNA: microRNA

Table 3: CT and delta CT values of salivary miRNA 31 in Control and OPMDs (PMD)

Sample	Gene	CT	Sample	Gene	CT	Delta CT
CON1	mir_16p	32.75	CON1	mir_31p	32.18	−0.58
CON2	mir_16p	32.80	CON2	mir_31p	40.00	7.20
CON3	mir_16p	33.54	CON3	mir_31p	31.32	−2.22
CON4	mir_16p	30.40	CON4	mir_31p	40.00	9.60
CON5	mir_16p	35.78	CON5	mir_31p	40.00	4.22
				Average	36.70	3.65
T1	mir_16p	30.89	T1	mir_31p	41.70	10.80
T2	mir_16p	30.25	T2	mir_31p	42.47	12.22
T3	mir_16p	29.69	T3	mir_31p	39.43	9.74
T4	mir_16p	29.30	T4	mir_31p	38.13	8.83
T5	mir_16p	31.24	T5	mir_31p	38.20	6.97
				Average	39.99	9.71

OPMDs: Oral potentially malignant disorders, MiRNA: microRNA



Graph 4: Comparison of salivary microRNA 21 and 31 expression in oral potentially malignant disorders

in oral squamous cell carcinoma when compared to healthy control. 11 miRNAs that are deregulated in oral squamous cell carcinoma were also found to be deregulated in OPMDs when compared to healthy control[9-14] and miRNAs, 21 and 31, have been analyzed repeatedly.[10,11,13,14]Three studies have unanimously proved miRNA 31 and miRNA 21[10,11,13] to be elevated in oral squamous cell carcinoma. The mean and SD calculated by Zahran *et al.*, in 2015, for miRNA 21 in oral cancer group was 0.155 with 65% sensitivity and specificity and AUC: 0.74.[10] Hung *et al.* reported 100% sensitivity with AUC as 0.73 for salivary microRNA 21. Similarly, Liu *et al.,* in 2012, have reported that salivary miRNA 31 was elevated in oral cancer with mean of −8.3, 100% specificity with AUC as 0.71 and no significant increase in oral verrucous leukoplakia cases.[13] Study done by Al-Malkey *et al.*, in 2015,[14] proved statistically significant rise in salivary microRNA 31 in oral cancer with mean of 26.379 versus control mean as 2.344. Hung *et al.* reported 100% sensitivity with AUC as 0.769 for salivary microRNA 31 and in this study both miRNA 21 and 31 were studied in tissue and saliva with significant increase in miRNA 31 in dysplastic epithelium. All six studies have used RT-qPCR to quantify salivary miRNA, while one of it uses nanostring miRNA expression assay along with RT-qPCR.[9-14] These six studies were assessed for their quality using QUADAS tool 2. Quality assessment of diagnostic accuracy studies has four domains, namely, patient sampling, index test, reference standard and flow and timing. Each of these domains had two to four questions which were answered as "yes," "no," or "unclear."[15] This data were fed into Review manager software, namely, in RevMan 5.3 to obtain a color-coded chart of risk of bias and applicability concern [Figure 6]. However, one study by Zahran *et al.* proved to be of low risk of bias having predetermined a cut off value for the miRNA before the onset of the study. The same study has proved that upregulated miRNA 21 and downregulated miRNA 145 are markers for detecting OPMDs with dysplasia, OPMDs without dysplasia, and oral squamous cell carcinoma.[10] In contrast to these findings, the present study results reveal statistically

Table 4: Salivary miRNA 21 and 31 fold expression in OPMDs (PMD)

Salivary MiRNA	Control Average CT and average delta CT	PMD Average CT and average delta CT	Delta CT ΔΔCt=ΔCt (PMD)–Ct (control)	Fold expression $2^{-\Delta\Delta Ct}$ P
21	33.75	36.84	6.56–0.69=5.87	−58.48
	0.69	6.56		*P* (0.016)
31	36.70	39.99	9.71–3.65=6.07	−67.18
	3.65	9.71		*P* (0.014)

OPMDs: Oral potentially malignant disorders, MiRNA: microRNA

significant downregulation of both miRNA 21 and 31 in test samples when compared to control and average CT values of salivary miRNA prove that comparatively miRNA 21 is better expressed in saliva than miRNA 31. Earlier studies were done in Caucasian, Asian, African, Taiwan, Iraq, and Arabic populations proving the demand for such diagnostic studies in Indian population.

Conclusion

The diagnostic study of expression of salivary miRNA in OPMDs has proved that salivary miRNA can be used as a biomarker as there is an expression of both reference gene [mir_16p] and target genes [mir_21p and mir_31p] in both control and in OPMDs. Down-regulated gene expression in Indian population is a significant racial difference as upregulation of these target genes have been studied in other populations such as Caucasian, Asian, African, Taiwan, Iraq, and Arabic. Statistical test of significance reveals that mir_31p ($P = 0.014$) and mir_21 p ($P = 0.016$) shows definitive down-regulated expression fold difference in OPMDs compared to control samples. The results of this pilot study proves the demand for expanding the research not only by increasing the sample size but also studying the expression in moderate and in severe dysplasia cases to analyze the correlation of expression fold of mir _21 p and mir_31p in varying degree of dysplasia cases.

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