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Expression profile of salivary micro RNA-21 and 31 in oral potentially malignant disorders

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Abstract: Oral potentially malignant disorders (OPMD) possess significant chances of malignancy conversion. In order to develop an early diagnostic tool, the present study evaluated the expression of miRNA-21 and 31 as salivary markers. The case-control study was carried out in 36 healthy participants as controls and in 36 patients who were newly diagnosed as OPMD having four different lesions including leucoplakia, oral sub mucous fibrosis (OSMF), oral lichen planus, and (OSMF) with leucoplakia. The samples were also classified as non-dysplastic, or with mild, moderate, and severe dysplasia according to their histopathological reports. The salivary miRNA-21 and 31 expressions were studied using real-time PCR. The statistical analysis was carried out using SPSS version 22. Salivary miRNA-21 (p-value = 0.02) and 31 (p-value = 0.01) were significantly upregulated in severe dysplasia compared with control. Among the different lesions, leucoplakia had significant upregulation of miRNA-21 and 31. miRNA-21 can be used as a diagnostic marker with specificity of 66% and sensitivity of 69%. The area under the ROC curve was 0.820 for miRNA-21 and 0.5 for miRNA-31, which proved that miRNA-21 is a better diagnostic marker than miRNA-31 for OPMD.

Keywords: Saliva; MicroRNAs.

Introduction

Oral cancer consists of tumors emerging in the lips, hard palate, upper and lower alveolar ridges, anterior two thirds of the tongue, sublingual region, buccal mucosa, retromolar trigone as well as floor of the mouth.[1] In India, oral cancer ranks in the top three of all cancers and is a high threat in low income groups with great exposure of tobacco chewing and inadequate access to new diagnostic equipment resulting in the increase of oral cancer incidence. Tobacco-associated oral lesions have various clinical presentations such as growth patterns and white or red patches. The red and white lesions have increased malignant potential. In 1978, WHO established the term precancer lesions and conditions, which was later changed to oral potentially malignant disorders (OPMD) in a workshop held in London in 2005.[2] The change in nomenclature from precancer to potentially malignant was because not all lesions develop into oral cancer while some have raised potential to develop in to oral

cancer; the word potential means the ability of being but not yet in existence.[3]

The prevalence of OPMD is 1–5% with rate of malignant transformation reported as 0.13–2.2% in the global scenario while it is somewhat greater in India with prevalence of OPMD reported as 2.5–8.4% with 17% malignant transformation rate. The conversion of these lesions into malignant lesions is dependent on the degree of epithelial dysplasia.[4] The transformation rate of OPMD with histopathological report of dysplasia into oral cancer is 36.4% and this stands for a fairly late phase in the multistage process of oral carcinogenesis.[5] The prevalence of oral cancer is increased in India primarily as a result of high tobacco chewing, which triggers a genetic damage.[6] Oral cancers are generally oral squamous cell carcinoma (OSCC) around non-keratinized epithelial lining of mouth, like tongue and floor of the mouth. An early medical diagnosis of the progression of OSCC is essential for the recovery from oral cancer.[7]

There are more than 10 types of OPMDs. The present study concentrated on three lesions: leukoplakia, oral submucous fibrosis (OSMF), and oral lichen planus with or without dysplasia. Leukoplakia is specified as a white patch that cannot be identified as any other lesion and with increased risk of malignant potential. OSMF is a persistent condition defined by fibrosis of the mucosa in the upper digestive tract. Oral lichen planus is a persistent condition mostly a result of the accumulation of T cells underneath the oral mucosa epithelium resulting in differentiation of stratified squamous epithelium, hyperkeratosis, and erythema with or without ulcer. The conversion of these lesions into malignant lesions generally depends upon the occurrence and degree of epithelial dysplasia.[4] The delay in the medical diagnosis of OPMD is a significant factor for the high prevalence of oral cancer. The screening of lesions by basic healthcare workers could be a beneficial approach to the early detection of cancer.

MicroRNAs (miRNAs) are a group of small RNAs involved in regulating the expression of protein coding genes.[8] miRNA have approximately 22 bp nucleotides that suppress the target mRNA by base pairing with the 3′untranslated region (UTR) and has both anticancer and cancer promoting activities. miRNAs revolutionized the field of molecular biology and have a vital role in the screening of early markers for cancer diagnosis,[9] playing an important role in tumor growth, invasion, angiogenesis, and immune cells invasion by controlling the expression of target mRNA.[10] miRNAs are encoded in the whole genome with a vast majority located in intergenic regions (anywhere between 57 and 69%), followed by intronic regions (~12 to 17%), exonic (~5%), long-noncoding (5%), and repeat regions (~8%). Nevertheless, around 50% of these genomic regions are frequently prone to alterations in various cancers and are collectively termed as cancer-associated genomic regions (CAGRs).[1] The presence of miRNA in oral saliva is one of the emerging tools to diagnose OSCC.[11] Among various miRNA, miRNA-31 and miRNA-21 are widely studied mRNAs, which can potentially act as biomarkers. miRNA-31 is highly upregulated and in cancer patients compared to the healthy control. It regulates the tumor suppressor gene and its knock down in lung cancer decreases the proliferation of cells and tumorigenicity.[12] miRNA-21 is another important biomarker highly upregulated in cancerous patient when compared to the healthy individuals. This miRNA helps the cells to escape from apoptosis and chemotherapy. The important function of miRNA-21 is regulating cell growth and proliferation, and upregulation of this gene is responsible for rapid cell growth.[12]

A systematic review on salivary miRNA in OPMD and oral cancer was carried out before beginning the research to evaluate the most commonly expressed ones and the diagnostic efficiency based on the ROC curve analysis. The results of the systematic review based on the risk of bias revealed that miRNA-21 is upregulated in oral cancer among various salivary miRNA. Few studies have been carried out to study the upregulation of miRNA-21 and 31 in salivary samples of oral cancer patients, which are highly upregulated in cancer patients. Both miRNAs-21 and 31 were significantly elevated in OPMD patients compared to control individuals with an AUC of 0.74 (95% CI, 0.60 to 0.89) and 0.76 (95% CI, 0.62 to 0.90), respectively.[13] miRNAs affect several cell-signaling pathways essential to carcinogenesis.[14] miRNA-31 was highly expressed

in saliva compared to serum samples and excision of oral cancer reduced its expression indicating that most of the upregulated salivary miR-31 came from tumor tissues.[15]

This research aimed to review the expression level of salivary miRNA-21 and 31 in OPMD and compare to healthy individuals, among various OPMD lesions, among various grades of dysplasia, and evaluate the most sensitive and specific miRNA in detecting early dysplastic changes in OPMD.

Methodology

The study was approved by the Institutional Ethical Committee, Department of Research, Saveetha University, Chennai, India (032/02/2017/IEC/SU). The informed consent was explained and the signed approval was obtained from all the participants. The inclusion criteria were patients suffering from OPMD including OSMF, oral leukoplakia, and/or oral lichen planus. The exclusion criteria were patients not willing to undergo biopsy and patients under treatment for OPMD. A form was prepared to document patients' demographic details and all patients were subjected to histopathological examination. Medical records such as histopathological reports were maintained confidential. The sample size was calculated to achieve a power of 80% using the Master Software version 2 using the formula

$$n = \frac{2S_p^2[Z_{1-\alpha/2} + Z_{1-\beta}]^2}{\mu^2_d}$$

where $S_p^2 = S1^2 + S2^2/2$, S1 is the standard deviation in the first group (3 SD = 2.94), S2 is the standard deviation in the second group (3 SD = 2.94), μ^2_d is the mean difference between the samples (2.975), α is significance level (0.5), and 1-β is the power (80%) for 2-tailed test. The mean difference between salivary miRNA-21 in OPMD with dysplasia and control was 2.975 as published by Zahran et al.[23] By using the formula, the required sample size per group obtained was 27. Thus, 36 patients newly diagnosed with OPMD based on the WHO diagnostic criteria were enrolled including 12 with OSMF, 8 with leukoplakia, 9 with oral lichen planus, and 7 with OSMF and leukoplakia. Saliva was collected from healthy samples (n= 36) and the expression of miRNA- 21 and 31 was assessed in both healthy subjects and OPMD cases.

Extraction of microRNA and reverse transcription

Saliva collection

Participants were asked to refrain from any food 2 hours prior to sample collection and from smoking or chewing tobacco one day before sample collection. Samples were obtained by requesting participants to avoid swallowing saliva for 5 minutes and expectorate all saliva in a tube. Saliva samples were collected in sterile DNAse/RNAse-free 50 mL falcon containers (cat#546041, Tarson, India), the containers were transported on ice to the laboratory within 3 h of collection and stored at 4ºC until use.

RNA extraction

Three hundred microliters of saliva was added to a fresh 1.5 mL sterile DNAse/RNAse-free microtubes (cat#500011, Tarson) and centrifuged at 12,000 rpm for 3 min at room temperature. Following centrifugation, 250 µL of the supernatant was transferred to a fresh microtube and subjected to miRNA extraction as per manufacturer's instruction (cat#740981.50, Machery-Nagel, Germany). MLP buffer (75 µL) was added to the saliva supernatant supplemented with 20 µL of protease (Mat#1016330, Qiagen, Germany), mixed, and incubated at room temperature for 3 min, which results in lysis of salivary proteins. Following lysis, 25 µL of MPP buffer was added and incubated at room temperature for 1 min, during which salivary proteins are denatured. Then, the lysate was centrifuged at 12,000 rpm for 3 min at room temperature to pellet the denatured proteins. After, 300 µL of the lysate was transferred to a fresh 1.5 mL sterile DNAse/RNAse-free microfuge tube and 300 µL of isopropanol was added to precipitate miRNA. The lysate along with the precipitated miRNA was transferred to a miRNA capture column and centrifuged at 12,000 rpm for 30 s to enable capture of the miRNA in the silica gel. Subsequently, the miRNA in silica gel was treated with RNAse-free DNAse (rDNase, cat#740963,

Machery-Nagel, Germany) to remove contaminating genomic DNA. Following this step, the miRNA in silica gel was washed twice with 500 µL of wash buffer and total miRNA was eluted in 20 µL of DNAse/RNAse-free water.

cDNA synthesis

Reverse transcription of miRNA was performed using miScript II RT Kit (Cat#218161, Qiagen) to synthesize complementary DNA (cDNA). Briefly, 12 µL of miRNA eluate was mixed with 4 µL of Hiflex buffer, 2 µL of nucleic mix (containing random hexamers for reverse transcription), 1 µL of RNAse inhibitor (cat#N2511, rRNAsin Ribonuclease Inhibitor, Promega, USA), and 2 µL of reverse transcriptase enzyme containing random hexamers for reverse transcription in a total volume of 20 µL. The mix was incubated at 37ºC for 60 min to allow reverse transcription reaction. The reaction was terminated by incubating the mix at 95ºC for 5 min.

Quantitative real time PCR

In order to quantitatively determine the copy numbers of each miRNA (relative to each other and among the samples), a standard curve was established with serial dilutions of 102 bp PCR product amplified from human beta-globin gene. The PCR was amplified with the following set of primers: forward GTG CAC CTG ACT CCT GAG and reverse CCT TGA TAC CAA CCT GCC CAG under the conditions of initial denaturation at 95°C for 10 min, amplification for 35 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s with a final extension at 72°C for 4 min. The PCR amplicon was gel purified (cat#NA1111, Sigma-Aldrich, USA) and eluted in 40 µL of elution buffer. To determine the concentration, 1 µL of the eluate was analyzed by Qubit fluorometer (Invitrogen, Austria) using QuantiFluor ONE dsDNA system (cat#E4871, Promega, USA). Number (or copies) of PCR amplicons present in ng of beta-globin gel eluate was determined by using the following formula.

$$\textit{Number of PCR amplicons} = \frac{\left(\frac{ng}{\mu L}\right) \times 6.022 \times 10^{23}}{(\textit{length of amplicon in base pairs}) \times 1 \times 10^{9} \times 650}$$

Based on the copies of PCR amplicons, serial dilutions were made to obtain concentrations from 1 x 10^6 to 1 x 10^1. These serially diluted samples were subjected to the amplification condition by initial denaturation in 95°C for 5 min, 40 cycles of amplification at 95°C for 5 s, and 60°C for 10 s in the presence of QuantiNova SYBR Green PCR Kit (Cat#208052, Qiagen) or SYBR Green Premix ExTaq Tli RNase H Plus (cat#RR820L, Takara, Japan) in Qiagen 5-plex rotor gene real time PCR system.

Real time PCR and melt curve analysis

In order to identify the quantitative presence of miRNA-21 and 31, the cDNA samples were subjected to real time PCR amplification with miRNA-21 (Hs_miR21_1_PR, miScript Precursor Assay, cat#MP00001498, Qiagen) and miRNA-31 (Hs_miR31_1_PR, miScript Precursor Assay, cat#MP00008757, Qiagen) specific primers. Control reaction for each sample was performed with U6 miScript Primer Assay (Hs_RNU6-2_1, cat#218193, Qiagen). All amplifications were performed in the presence of QuantiNova SYBR Green PCR Kit (Cat#208052, Qiagen) in a 20 µL reaction. The following amplification condition was used for quantitative determination of miRNA-21, miRNA-31, and control U6 miRNAs: after an initial denaturation at 95°C for 4 min to activate the taq polymerase, the samples were subjected to 40 cycles of 95°C for 15 s and 60°C for 30 s with capture of fluorescence after extension. Following amplification, the normalized amplification curves were quantified in relation to a standard curve by the built-in software in Rotor Gene Q real time system (Qiagen) and concentration of miRNA-21 and 31 in each sample was expressed as copies per µL. To confirm the specificity of amplifications, the samples were subjected to melt curve analysis, which involved a ramp step that ranged between 60 and 95°C with an initial hold for 90 s followed by a rise of 1°C at each step with a 5 s hold to enable the melting process. Analysis of melt curve showed distinct peak intensities for each of the three miRNAs between 75 and 85°C.

Statistical analysis

The difference of salivary miRNA-21 and 31 in varying OPMD lesions and grades of dysplasia were

analyzed by one-way ANOVA and Student's Newman Keul's multiple comparison tests. Correlation between the different parameters such as chewing and smoking habit intensity and duration with respect to expression profile of salivary miRNA-21 and 31 was calculated by the bivariate Pearson's correlation test and P values of 0.0001 and 0.05 were considered highly significant and significant, respectively. Linear regression equation was used to predict the expression of salivary miRNA with respect to smoking and chewing habit duration. A ROC curve with sensitivity and specificity was constructed for salivary miRNA-21 and 31.

Results

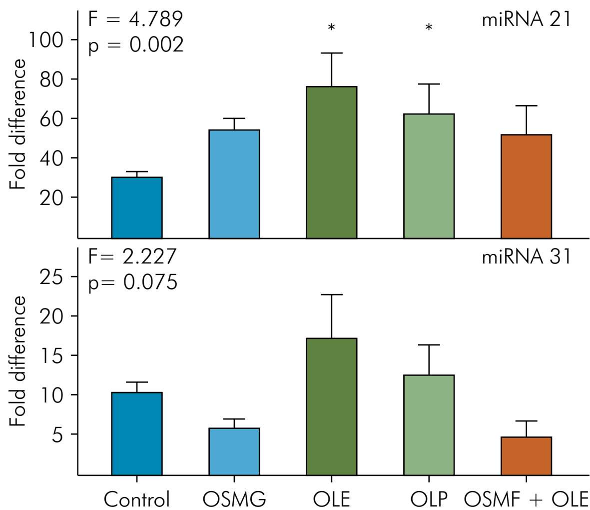
In the present study, we evaluated the expression level of salivary miRNA-21 and 31 in OPMDs. Expressions were calculated as fold difference in relation to the housekeeping gene U6. Table 1 reveals the mean (SE) of fold difference of miRNA-21 and 31 in various OPMD lesions. Significant difference in the mean fold difference of miR-21 among participants with oral leukoplakia and controls and among participants with oral lichen planus and controls with a fold-increase of 2.44 times and 2.03 times, respectively, and a p value of 0.002. Though there was no statistically significant difference in the mean fold difference of miRNA-31 among the study group and control group with a p value of 0.075, upregulation was observed more in oral leukoplakia followed by oral lichen planus (1.6 and 1.2 times respectively) when compared to OSMF and OSMF with oral leukoplakia, similar to miRNA-21 where upregulation was highest in oral leukoplakia followed by oral lichen planus when compared to OSMF. Figure 1 reveals the fold difference expression profile of miRNA-21 and 31 in various lesions of OPMD.

In 36 clinically and histopathologically diagnosed OPMDs, 15 cases were reported as no dysplasia and 22 cases as dysplasia (9 mild dysplasia, 7 moderate dysplasia, and 5 severe dysplasia). Table 2 reveals the mean (SE) fold difference of miRNA-21 and 31 in various degrees of dysplasia. Statistically significant difference was found in the mean fold difference of miRNA-21 values among participants with no dysplasia and controls and among participants with severe dysplasia and controls with a p value of 0.011. However, no statistically significant difference was found in the mean fold difference of miRNA-21 among participants with mild and moderate dysplasia when compared to controls. A statistically significant difference was found in the mean fold difference of miRNA-31 among the study group with severe dysplasia and control group with a p value of 0.001. No significant difference was found for miRNA-31 between control group and no dysplasia, mild, and moderate dysplasia groups. Figure 2 reveals the fold-difference expression profile of miRNA-21 and 31 in various grades of dysplasia.

A significant correlation was found only between smoking (r = 0.636 and p value of 0.003) and chewing habits duration (r = 0.695 and p value of 0.008) and fold difference of miRNA-31. Hence, linear regression equation was only derived for miRNA-31 with habit

Table 1. Mean (SE) of fold difference of salivary miRNA-21 and miRNA-31 in various.

S. N°	Parameters	Groups	N	Mean	SEM
1.	miR-21	Control	36	30.665	3.100
		OSMF	12	54.029	6.792
		Oral leukoplakia	8	75.026	18.406
		Oral Lichen planus	9	62.312	15.705
		OSMF with oral leukoplakia	7	51.403	15.420
2.	miR-31	Control	36	10.472	1.550
		OSMF	12	5.965	1.310
		Oral leukoplakia	8	17.258	5.793
		Oral Lichen planus	9	12.616	4.017
		OSMF with oral leukoplakia	7	4.985	1.998



Control (n = 36); OSMF: oral submucous fibrosis (n = 12); OLE: oral leukoplakia (n =08); OLP: oral lichen planus (n = 9); OSMF with OLE (n = 7). The data were analyzed by one-way ANOVA with Student Newman Keul's multiple comparison. *Significantly different from control.

Figure 1. Mean of fold difference of miRNA-21 and miRNA 31 in OPMD.

duration. Tables 3 and 4 reveal the linear regression equation for prediction of miRNA-31 expression based on the years of smoking and chewing tobacco duration. No statistically significant correlation was found between smoking (r = 0.373 with a p-value of 0.116) and chewing duration (r = 0.266 and p-value of 0.379) and miR-21 fold-difference. Figures 3 and 4 show the scatter plots of miRNA-21 and 31expression with intensity and duration of smoking and chewing tobacco. The area under the miRNA-21 ROC curve was 0.82 with 69% sensitivity and 66% specificity and significant p value of 0.0001 while the area under the miRNA-31 ROC curve was 0.51 with 36% sensitivity and 40% specificity and non-significant p value of 0.899. Figure 5 (A and B) shows the ROC curve analysis of salivary miRNA-21 and 31.

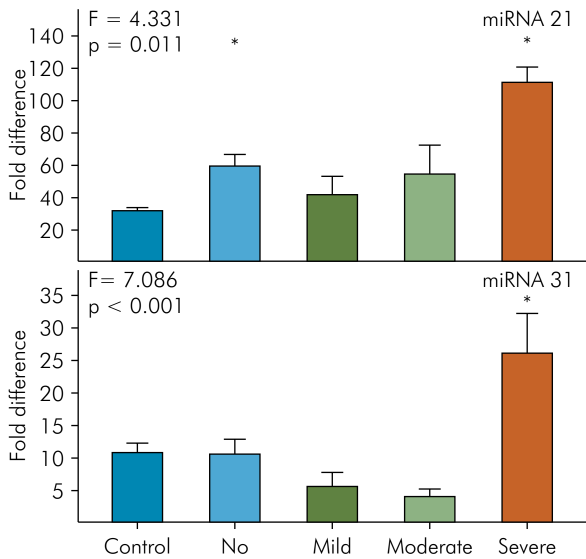
Discussion

Deregulation of miRNAs is known to be associated with many diseases. Salivary miRNAs are stable and can have potential use in oral cancer detection.[20] Cancer-associated miRNAs are generally classified into 2 groups, the onco-miRNAs and tumor suppressor miRNAs. miRNA-21 promotes cancer development via regulating cell transformation, proliferation, cell cycle, apoptosis, and metastasis.[16] In the present study miRNA-21 expression was upregulated in patients with leukoplakia and severe dysplasia. Previous studies on oral premalignant lesions revealed upregulation of miRNA-21 as an early event in cancer development[17] and to be strongly correlated with the progression of pre-malignant leukoplakia to invasive oral carcinoma.[18] The expression profile of miRNA-21 in tongue squamous cell carcinomas was associated with poor prognosis and was reported as highly expressed in advance tumor (~39.51-fold higher) when compared to the early stages of cancer.[19]

Studies on salivary miRNA expression in OPMD are sparse as only in 2009 miRNA expression studies in oral cancer began.[20] The systematic review conducted before selecting the miRNAs for the present research included six studies on salivary miRNAs with one

Table 2. Mean (SE) of fold difference of miRNA-21, miRNA-31 in various degrees of dysplasia.

S. N°	Parameters	Groups	N	Mean	SEM
1.	miR-21	Control	36	30.665	3.100
		No dysplasia	15	57.879	8.314
		Mild dysplasia	9	41.313	11.604
		Moderate dysplasia	7	54.030	17.618
		Severe dysplasia	5	110.200	10.715
2.	miR31	Control	36	10.472	1.550
		No dysplasia	15	10.190	2.624
		Mild dysplasia	9	5.446	2.102
		Moderate dysplasia	7	3.838	1.229
		Severe dysplasia	5	25.869	6.122



Control (n = 36); No dysplasia (n =1 5); mild dysplasia (n = 9); moderate dysplasia (n = 7); severe dysplasia (n = 5). The data were analyzed by one-way ANOVA with Student Newman Keul's multiple comparison.

Figure 2. Mean of fold difference of miRNA-21 and miRNA-31 in varying grades of dysplasia.

study exclusively done in OPMD (Hung et al. 2016)[15], two studies done exclusively in oral cancer,[20,21] and three studies done in both OPMD and cancer.[8,22,23] Around 21 miRNAs have been established in OPMD and oral cancer in the six studies on salivary miRNA, including 78 OPMD participants and 167 oral cancer participants, in which 11 miRNAs deregulated in OSCC were found to be deregulated in OPMD.[15,22,23]

Salivary miRNA-21 was studied by Zahran et al.[23] in 20 OPMD with dysplasia and 20 OPMD without dysplasia against 20 healthy controls and 20 disease controls, and by Hung et al. (2016)[15] in 20 saliva samples and 46 tissue samples from patients against 24 healthy controls. The mean value for miR-21 was only mentioned in the Zahran et al.[15] study as 3.7, who concluded that there is a four-fold increase in miRNA-21 in OPMD compared to the controls with area under the ROC curve of 0.73 with 65% sensitivity and specificity. ROC analysis was reported as 0.74 with 100% sensitivity in the study done by Hung et al.[15]. The area under the ROC curve for miR-21 in the present study was 0.82 with 66% sensitivity and 69% specificity with a 2-fold increase, which is a reasonable cutoff point for predicting OPMDs cancer transformation.

miRNA-31 was upregulated almost 20 times in cancerous tissue compared to healthy adjacent tissue and it mediates oncogenesis by targeting a molecule that inhibits hypoxia inducing-factor in oral cancer.[8]

Salivary miRNA-31 was studied by Liu et al.[8] in 10 verrucous leucoplakias, 45 OSCC, and 24 healthy controls and it was concluded that the mean value for miRNA-31 was 8.3 with area under the ROC curve of 0.71 and 100% specificity. A study conducted in 35 OSCC and 20 healthy controls by Al-Malkey et al.[21] found a cutoff value for miRNA-31 of 6.623 with mean of 26.4 and concluded that miRNA-31 inhibits negative regulators of cancer pathway and promotes cell proliferation. A study done by Hung et al.[15] in 20 saliva samples of OPMD did not mention the mean value but based on an AUC of 0.74 concluded that miRNA-31 was significantly upregulated in OPMD compared to control. In the present study, miRNA-31 was upregulated in oral leukoplakia, oral lichen planus, and in severe dysplasia alone group with 0.6 increase rate, 36% sensitivity, and 40% specificity.

A study on alteration of miRNA expression in oral keratinocytes reported that there is a 3-fold

Table 3. Linear regression equation for prediction of miRNA-31 expression based on the years of tobacco smoking.

Variables	Model	Model fit (R square)	a (Intercept)	b (regression coefficient)	p-value
Smoking duration and microRNA31	Bivariate - Enter	0.405	0.723	0.636	0.003#

miR-31 = 0.723 + 0.636 (n° of years of Smoking) (y = a + bx). #Bivariate Linear regression, Sig at ≤ 0.05 level.

Table 4. Linear regression equation for prediction of miRNA-31 expression based on the years of tobacco chewing.

Variables	Model	Model fit (R square)	a (Intercept)	b (regression coefficient)	p-value
Chewing tobacco and microRNA31	Bivariate - Enter	0.483	0.902	0.695	0.008#

miR-31 = 0.902 + 0.695 (no: of years of use of smokeless tobacco) (y = a + bx). #Bivariate Linear regression, Sig at ≤ 0.05 level.

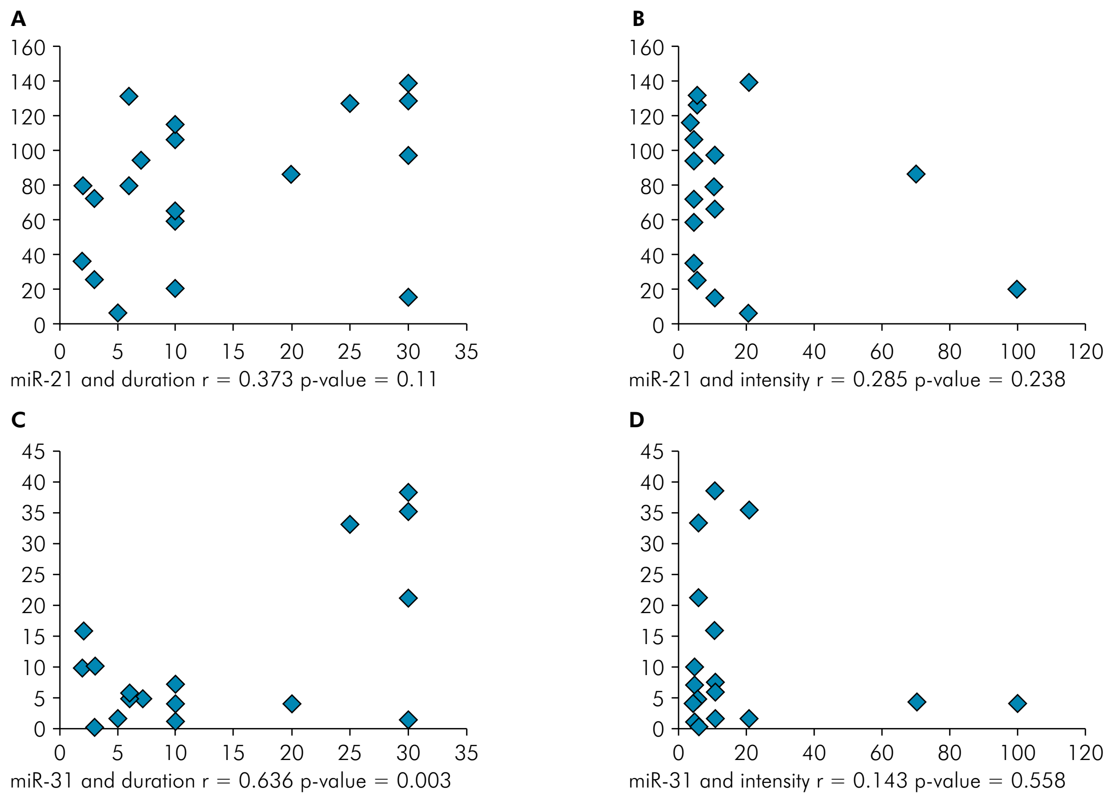


Figure 3. Correlation between tobacco smoking intensity and duration with fold difference of miRNA-21 and miRNA-31.

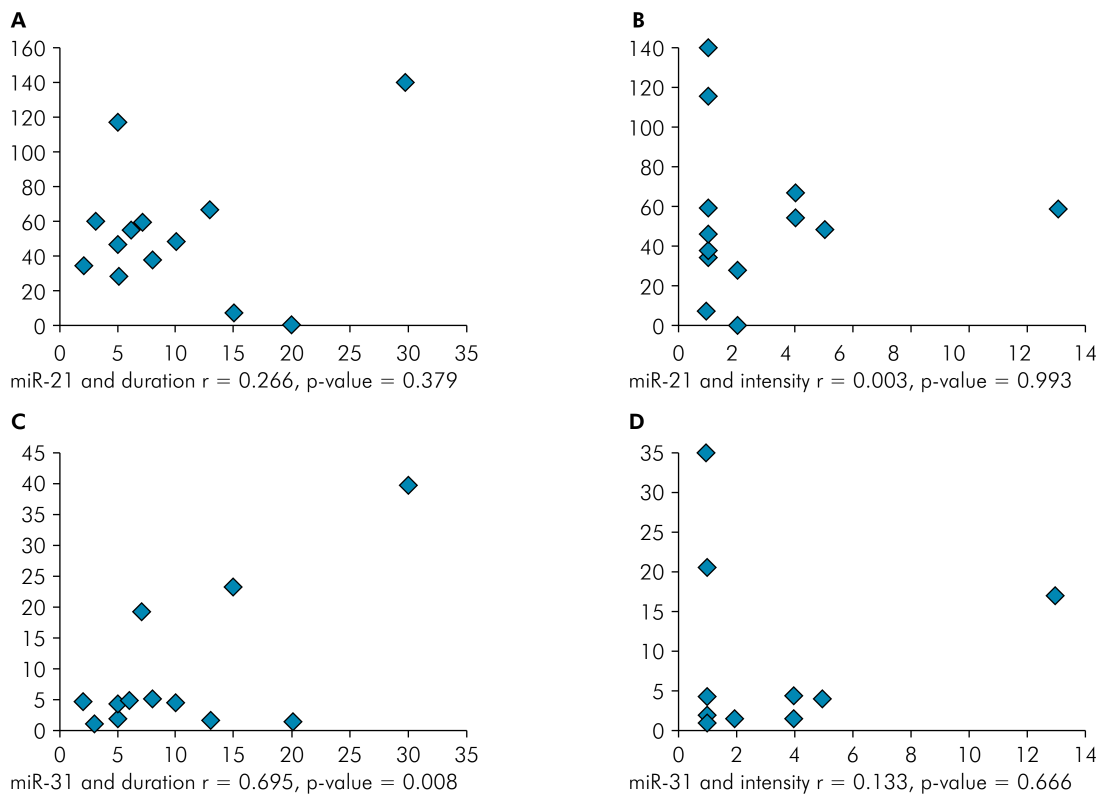


Figure 4. Correlation between tobacco chewing intensity and duration with fold difference of miRNA-21 and miRNA-31.

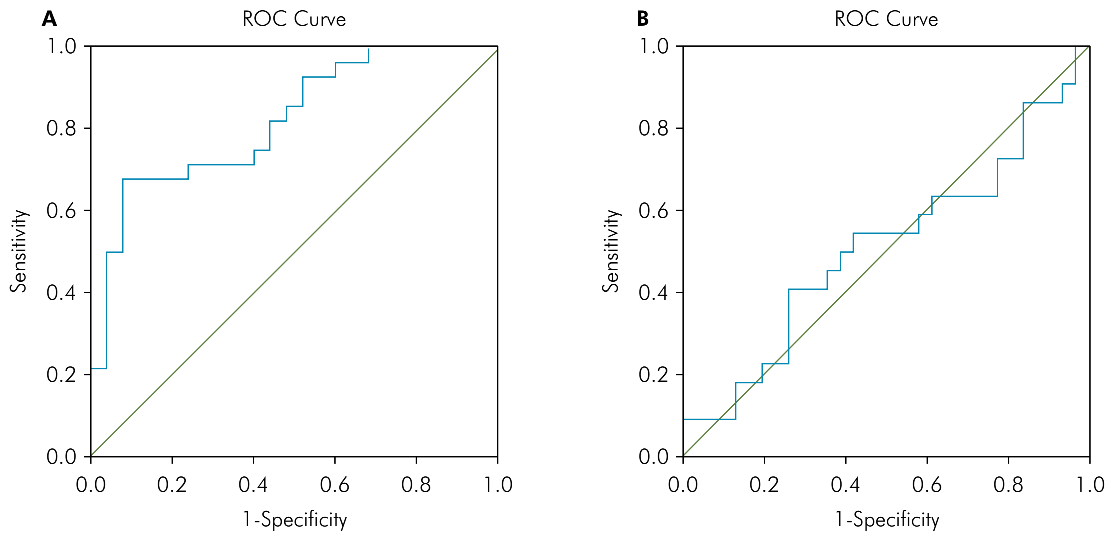


Figure 5. A: ROC curve for miRNA-21; B: ROC curve for miRNA-31.

increase in miRNA-31 expression by tobacco smoking and chewing frequency.[24] The association between tobacco smoking and chewing duration and fold difference of miRNA-31 expression of the present study is consistent with the above-mentioned study as it found a significant association between them with p value of 0.003. A linear regression equation was obtained based on the positive correlation results of the present study for smoking duration and tobacco chewing duration with miRNA-31. On substituting the number of years of habit duration using this equation, a predicted value of fold difference for miRNA-31 can be obtained. This helps improve preventive measures through health education to individuals with long habit duration.

Conclusions

Upregulation of miRNA-21 and miRNA-31 was significant in oral leukoplakia and oral lichen planus, and was minimal in OSMF and OSMF with leukoplakia groups. There was a significant difference in miRNA-21 expression between control and OPMD when compared to miRNA-31. There was a significance association between salivary miRNA-21 with severe dysplasia, with a 3.6-fold increase, while an association of salivary miRNA-31 with severe dysplasia alone was observed, with a 2.5-fold increase. Previous research focused in biomarkers for oral cancer, thus we concentrated in OPMD lesions. Salivary miRNA-21 can be utilized as a potential adjuvant diagnostic biomarker to evaluate very early malignant changes in OPMD; its potential was much higher when compared to salivary miRNA-31.

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