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*Original Research Article*

# Expression of Salivary miRNA-31 in Oral Submucous Fibrosis



## Abstract

**Objective:** The present study aimed to evaluate the salivary miRNA31 expression in controls and cases and associate miRNA31 levels with clinical parameters of oral submucous fibrosis.

**Methods:** This case control study was conducted in a hospital setup. A total of 50 individuals participated in the study with 25 subjects in group I (healthy individuals) and 25 subjects in group II 25 diagnosed cases of (oral submucous fibrosis). The sample size was calculated with open Epi version 3.01. A detailed assessment of clinical parameters of oral submucous fibrosis was made. Unstimulated saliva samples were collected from all study subjects meeting the inclusion criteria and analysis of saliva samples was done by qRTPCR.

**Results:** The results showed high expression levels of miRNA31 in oral submucous fibrosis as compared to the control group. The study demonstrated significantly higher median fold change of miRNA-31 expression level in OSMF patients as compared to the participants in the control group. Correlation between age of patients and miRNA31 fold change was discerned using the Spearman rank test that demonstrated a non-significant negative correlation.

**Conclusion:**

Increased expression levels of miRNA 31 among oral submucous fibrosis as compared to the control group make it a promising salivary biomarker that detects oral submucous fibrosis at an early stage of the disease.

**Keywords:** Saliva, miRNA31, Salivary biomarker, oral submucous fibrosis, qRTPCR

## Introduction

Oral submucous fibrosis (OSMF) is a premalignant condition that is characterized by the formation of excess fibrous connective tissue. It is mainly related to the usage of areca nut exclusively or in amalgamation with betel quid/tobacco and its byproducts. It is a high-risk precancerous condition characterized by excessive fibrotic changes in the lamina propria and the

associated connective tissue of buccal mucosa[1].The epidemiological researches reported that the disease is largely found in Asian countries like India, Pakistan, Bangladesh, Taiwan, Sri Lanka, and China where the frequency of areca nut use has been seen to be considerably greater[2-3].

The fatality rate of OSMF considerably rises because of its fast conversion to oral squamous cell carcinoma[4].The ethnicity and geographical region influence the prevalence of OSMF and are directly linked with dietary habits, lifestyle, and traditions[5-6].Risk factors that cause OSMF involve chronic inflammation leading to OSMF, along with dietary intake and nutritional status (deficiency of vitamin B, C, and iron), cancer-causing factors (consumption of betel nut and tobacco), alcohol, and intake of spicy diet[7].

Recently, biological indicators have been recognized by the present biological procedures, like promoter methylation, cytological features, polymorphism, non-coding RNAs, microRNA, mRNAs, and protein and trace elements in a serum, tissue biopsy, and saliva are used as potential biomarkers for OSM[8].The usage of serum, tissue, cytology, and saliva for investigation has distinctive benefits. Besides the analysis of OSMF, one or many biological markers expression ratios may also be utilized as an innovative OSMF staging process for enhancement of evaluation index of OSMF, calculates OSMF changes to malignant tumor index, and envisages the accurate treatment [9].

Early identification of the OSMF helps provide the immediate and right treatment. Numerous OSMF stages and grades have been classified and recorded earlier, such as clinical, functional, and histological. Certain staging and grading systems are applied by the physician for the detection and management of OSMF [10-11]. The unstimulated saliva contains a large number of proteins, bioactive peptides, nucleic acids, and electrolytes secrete from the major salivary glands (parotid glands, submandibular gland, sublingual glands), and several minor glands [12]. Saliva has several benefits over serum and tissue fragments. One of the most attractive features is the noninvasive method that combines with the easy method of collection and storage, making it a beneficial tool. Modern technologies have proven their efficiency and shown a large number of salivary biomarkers that are associated with numerous general and oral diseases[13].Hundreds of genes in genetic information in an organism specify the genetic code on minute functional RNA molecules collectively called miRNAs and are present in the blood, saliva, and normal tissues[14].

This research is intended to investigate whether an increased quantity of salivary miRNA-31 can be used as a biological marker for the initial detection of OSMF, along with malignant changes from OMFS to OSCC. Furthermore, it will help future clinicians and researchers in early detection, categorizing, and improving the management of patients of OSF.

## Materials and Methods

This case control study was conducted in 6 months period at the Department of Molecular Genetics, Ziauddin University in Karachi, Pakistan, after the approval from the ethical review committee of, Ziauddin University, Karachi (Reference code:3011220SKOM). Patients visiting as an outpatient in the department of Oral diagnosis of Ziauddin University and Altamash Institute of Dental Medicine diagnosed with oral submucous fibrosis were enrolled. Written informed consent from all participants was taken.

Analysis of saliva samples was done by qRTPCR at the North campus of Ziauddin university hospital, Karachi. The sample size was calculated using the open Epi version 3.01 software. The power of the test was taken as 90% and the confidence of interval was considered as 95%, the sample size came out to 6 per group which was increased up to 25 per group. In this study, a non- probability consecutive sampling technique was used.

## Sample Selection

50 individuals were randomly selected for this research from the population of Karachi, with the following division: Group 1 (n=25) 25 controls as clinically healthy persons of both gender and Group 2(n=25) 25 diagnosed cases of oral submucous fibrosis and were then graded according to the criteria mentioned in (Table 1). Fresh saliva from inducted individuals was collected in a 50ml sterile corning tube and stored at -80°C for further processing.

## Inclusion Criteria for Cases and Controls

Participants of both genders between the age of 18 to 70 years and clinically diagnosed cases of OSMF were selected in cases group while healthy individuals with no history of any other oral lesion were taken as controls.

## Exclusion Criteria for Cases and Controls

Participants with a history of hypertension, diabetes, cancers other than the oral cavity, pregnant females, and known cases of psoriasis, systemic lupus erythematosus, and rheumatoid arthritis were excluded from the study.

**Table 1:** Clinical Parameters of Oral Submucous Fibrosis

| GRADE | PARAMETERS |
|---|---|
| **Grade 1** | Involvement of < one-third of the oral cavity. Mild blanching, burning feeling, ulceration. Mouth opening not more than 35mm. |
| **Grade 2** | Involvement of one-third to two-thirds of the oral cavity, blanching, marble appearance, fibrotic band. Mouth opening between 25 to35mm. |
| **Grade 3** | Involvement of > two-third of the oral cavity, severe blanching, broad fibrotic bands at cheeks and lips, and stiff mucosa. Mouth opening 15 to 25mm. |
| **Grade 4** | Leukoplakia changes, Erythroplakia ulcerating, and suspicious malignant lesion. Mouth opening <15mm. |

## Statistical Analysis

All statistical analysis was performed using SPSS- 24. Mean and standard deviation was calculated for numerical data. Chi-squared test was used for the association of miRNA31 and case group. Further, frequency/percentage were calculated for categorical variables. To assess the mean difference of miRNA31 between case group one-way ANOVA was used. *p*-value < 0.05 was considered statistically significant. Correlation between age of patients and miRNA31 fold change was detected using the Spearman rank test (**Figure 1**) that demonstrated an insignificant negative correlation ($r= -0.236$, $p>0.05$).

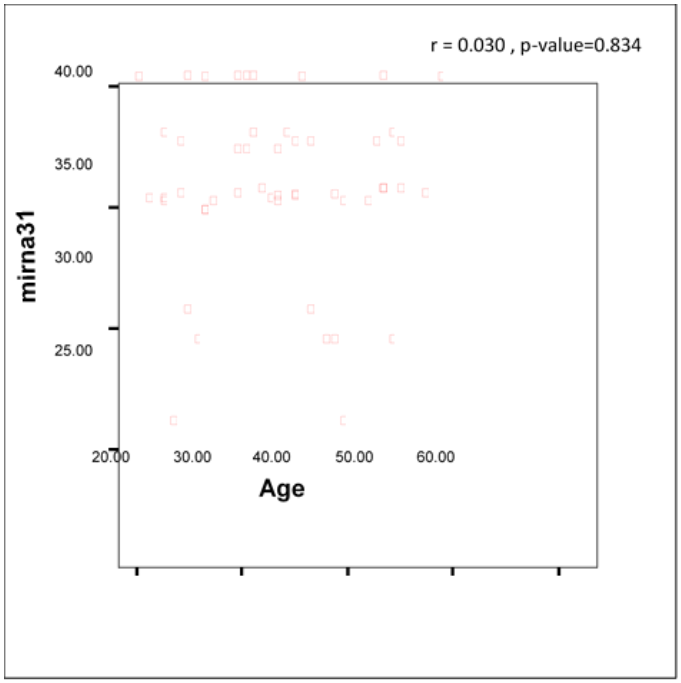


**Figure 1:** spearman correlation between age of patient and mirna31-fold

## Results

Table 2 illustrates the sociodemographic details of the study population. In the control group, 18(72%) of individuals were males and 7(28%) were females, while in the oral submucous fibrosis group 18(72.0%) were males and 7(28.0%) were females. The mean age of individuals was reported were 32.6±8.2 years (age range 20–58 years) in the healthy or control group and 36±7.9 years (age range 25–54 years) in the oral submucous fibrosis group.

When the observed frequency of betel nut chewing habits in oral submucous fibrosis, most of them 24(96%) were habitual to chew betel nut in the oral submucous fibrosis group. Most of the patients were smokers 17(68.0%) in oral submucous fibrosis while 8(32.0%) were non-smokers in oral submucous fibrosis. Based on the clinical staging of oral submucous fibrosis, 16 (64.0%) cases were presented in stage 2, 7(28.0%) were in stage 3 whereas only 2(8.0%) were reported in stage e1, as shown in (Table 3). Regarding the status of oral submucous fibrosis, the mean of habit duration was 14.1±6.1 years; the mean frequency was reported 11.7±3.1 per day, and mean of mouth opening was documented 26.2±7.8 mm in the oral submucous fibrosis group, as shown in (Table 4).

**Table 2:** Demographic characteristics of study subjects.

| VARIABLES | | Group 1 (Controls) | | Group 2 (Cases) | |
|---|---|---|---|---|---|
| | | Healthy | | OSMF | |
| | | Frequency (*n*) | Percentage *%* | Frequency (*n*) | Percentage *%* |
| **Gender** | Male | 18 | 72.0 | 18 | 72.0 |
| | Female | **7** | 28.0 | 7 | 28.0 |
| | **Total** | **25** | **100** | **25** | **100** |
| **Age** | Mean ± SD | 32.6±8.2 | | 36±7.9 | |
| | Min, Max | 20.0 | 58.0 | 25.0 | 54.0 |
| | **Total** | **25** | **100** | **25** | **100** |
| **Marital Status** | Married | 4 | 23.5 | 14 | 70 |
| | Unmarried | 13 | 76.5 | 6 | 30 |
| | **Total** | **17** | **100** | **20** | **100** |
| **Qualification** | Below 10th grade | 2 | 8.0 | 5 | 20.0 |
| | Below 12th grade | 3 | 12.0 | 4 | 16.0 |
| | Graduate | 14 | 56.0 | 15 | 60.0 |
| | Housewife | 0 | 0.0 | 0 | 0.0 |
| | Non educated | 1 | 4.0 | 0 | 0.0 |
| | Postgraduate | 5 | 20.0 | 1 | 4.0 |
| | **Total** | **25** | **100** | **25** | **100** |
| **Ethnicity** | Baloch | 0 | 0.0 | 1 | 4.0 |
| | Pathan | 1 | 4.0 | 2 | 8.0 |
| | Punjabi | 4 | 16.0 | 8 | 32.0 |

| | | | | | |
|---|---|---|---|---|---|
| | Sindhi | 3 | 12.0 | 0 | 0.0 |
| | Siraiki | 7 | 28.0 | 5 | 20.0 |
| | Urdu Speaking | 10 | 40.0 | 9 | 36.0 |
| | **Total** | **25** | **100** | **25** | **100** |



**Table 3:** Median Fold change of MicroRNA-31 associated with different variable in OSMF Group

| VARIABLES | ***n*** | **Median fold** | ***p*-value** |
|---|---|---|---|
| 1. **STAGING OF OSMF** | | | |
| **Stage 1** | **2** | **8.0** | 0.46 |
| **Stage 2** | **16** | **64.0** | |
| **Stage 3** | **7** | **28.0** | |
| **Total** | **25** | **100.0** | |
| 2. **SMOKING STATUS** | | | |
| **Smoker** | **17** | **68.0** | 0.3 |
| **Non-smokers** | **8** | **32.0** | |
| **Total** | **25** | **100.0** | |
| 3. **BETEL QUID/PAN & TOBACCO HABIT** | | | |
| **Yes** | **24** | **96** | 0.67 |
| **No** | **1** | **4** | |

| | | | |
|---|---|---|---|
| **Total** | **25** | **100.0** | |

**Table 4:** Mean of duration and frequency of consumption and mouth opening in oral submucous fibrosis

| Status | | n | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|---|
| **OSMF** | Duration in years | 25 | 2.0 | 27.0 | 14.1 | 6.1 |
| | Frequency per day | 25 | 5.0 | 18.0 | 11.7 | 3.1 |
| | Mouth Opening (mm) | 25 | 14.0 | 40.0 | 26.2 | 7.8 |



## Discussion

Biomarkers play an important role in the early and confirmatory diagnosis of many neoplasms. In recent years this field of medical science has taken a lead in the detection of incognito neoplasms leading to prompt treatment and better outcomes. Owing to advancements in biotechnology and molecular biology, research on molecular biomarkers has turned out to be useful in the diagnosis and determination of the prognosis of oral neoplasms. This study was designed to discern the effectiveness of microRNA31 as a salivary biomarker for diagnosis of one of the most common potentially premalignant disorders in South Asia, oral submucous fibrosis. For this purpose, 50 participants were enrolled in this study, which entailed 25 in the control group and 25 in OSMF. The high percentage of males in the diseased group (OSMF) as compared to the females. A study by Rahul on the Indian Population also showed the prevalence of OSMF to be higher in males as compared to females [15]. High consumption of tobacco and alcohol, nonchalant attitude towards getting a suspicious lesion diagnosed by a clinician, unconcerned about aesthetically unpleasant lesions, and greater tendency to pursue field jobs might be a few reasons why OSMF was shown to be higher in males as compared to females in our study [16].

Furthermore, for ethnicity status Urdu speaking were most prevalent in both groups (40% in the control group and 36% in the OSMF group. The second most prevalent ethnicity was the Punjabi community, which was highest in the OSMF group (32.0%). Most of the patients were graduates; 56% and 60.0%, in control and OSMF groups, respectively. Results from our study showed that the mean age of participants was 36 in OSMF group as compared to another India-based study reported that the prevalence of oral malignancies was highest in the 50 to 60 years age group [17]. All these studies are inconsistent with the findings of our study. Whereas a study on the Israeli Arab population showed that most (53.7%) of the diagnosed oral cancer patients were more than 54 years of age [18]. The dissimilarity of our findings with these studies might

be due to genetic, environmental, and habitual variations. In our study, consumption of betel nut was highest in the OSMF group (96%). Furthermore, a high percentage of patients were smokers in OSMF (68.0%) and control (32.0%) groups, respectively. In some Asian countries, the habits of tobacco chewing and betel quid usage are ingrained in cultural practices of a few ethnic groups and they have been documented as cardinal risk factors for OSMF [19]. The high percentage of patients with delinquency towards such hazardous habits is probably related to the early commencement of consumption of these products due to ease of availability at low cost. Multiple studies have reported that a greater risk of OSMF occurs in patients who chew tobacco and keep it in their mouth for a longer duration [20].

The differences could be related to genetic variations, chemical and environmental factors and maintenance of hygiene, and prevalence of viral infection such as HPV, causing cancer production and associated mucosal changes. The clinical behavior of oral cancer might be affected by factors such as tumor recurrence and poses a significant hurdle for early diagnosis of the disease; thus, reliable biomarkers are required for detection of oral potentially malignant and malignant diseases as well as for post-therapeutic follow-up. Saliva, unlike other body fluids, is in constant contact with all the tissues of the oral cavity, and is an excellent source for contemplating and deciphering the pathophysiological state of oral tissues. MicroRNAs (miRNAs) are short non-coding RNAs, that regulate cellular pathways and transcriptome. They have recently been used in cancer genomics and cancer diagnostics. miRNAs have been posited to be of significant diagnostic value as they can abet early detection of many cancers, as studies have affirmed miRNA's cardinal role in various phases of cancer development, progression, and prognosis [21-22]. The current study demonstrated significantly higher median fold change of miRNA-31 expression level in OSMF patients as compared to the participants in the control group. Correlation between age of patients and miRNA31 fold change was discerned using the Spearman rank test that demonstrated a non-significant negative correlation ($r=-0.236$, $P>0.05$). Li confirms in his study that miR-31 has been detected in malignancies of lung cells. His findings further suggested that miR-31 may play a vital role in oral cancer development [23].

## Conclusions

OSMF is the most common oral potentially premalignant disorder in Pakistan thus we also discerned the expression level of salivary miRNA-31 in OSMF utilizing qRT-PCR.Our study concluded that there is a strong association between the expression levels of miRNA-31 and the disease status of the subject. It might also abet future clinicians in early, efficient, less time consuming and relatively less discomfortable detection of OSMF by utilizing saliva diagnostics in discerning the level of miRNA-31.

## Limitations

The findings of our study should be formulated in light of two significant limitations. First, due to the small sample size, the results could not be generalized to a larger population. Second, the overall number of studies relevant to the expression of salivary miRNA-31 in oral submucous fibrosis is limited. To overcome these obstacles and confirm our results, other longitudinal and clinical-trial studies with larger sample size are required to determine expression of salivary miRNA-31 in oral submucous fibrosis.

## Ethical Approval

IRB: Approved by ethical review committee of, Ziauddin University, Karachi (Reference code:3011220SKOM).

## References

[1] Dionne KR, Warnakulasuriya S, Zain RB, Cheong SC. Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. Int J Cancer. 2015 Feb 1;136(3):503-15. doi: 10.1002/ijc.28754. Epub 2014 Feb 11. PMID: 24482244.

[2] Mathew AL, Pai KM, Sholapurkar AA, Vengal M. The prevalence of oral mucosal lesions in patients visiting a dental school in southern India. Indian J Dent Res. 2008;19(2):99–103. doi: 10.4103/0970-9290.40461.

[3] Sultana, Nishat, Shambulingappa Pallagatti, and Ali Imam Mohamed. "P53 expressions in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma." 2(1):9.

[4] Hosein M, Mohiuddin S, Fatima N. Association Between Grading of Oral Submucous Fibrosis with Frequency and Consumption of Areca Nut and Its Derivatives in a Wide Age Group: A Multi-centric Cross-Sectional Study from Karachi, Pakistan. J Cancer Prev. 2015 Sep;20(3):216-22. doi: 10.15430/JCP.2015.20.3.216. PMID: 26473161; PMCID: PMC4597811

[5] Chattopadhyay E, Singh R, Ray A, et al. Expression deregulation of mir31 and CXCL12 in two types of oral precancers and cancer: importance in progression of precancer and cancer. Sci Rep. 2016; 6:32735. Published 2016 Sep 6. doi:10.1038/srep32735

[6] Tilakaratne WM, Ekanayaka RP, Warnakulasuriya S. Oral submucous fibrosis: a historical perspective and a review on etiology and pathogenesis. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016 Aug;122(2):178-91. doi: 10.1016/j.oooo.2016.04.003. Epub 2016 Apr 19. PMID: 27422417

[7] Guruprasad R, Nair PP, Singh M, Singh M, Singh M, Jain A. Serum vitamin c and iron levels in oral submucous fibrosis. Indian J Dent. 2014 Apr;5(2):81-5. doi: 10.4103/0975-962X.135266. PMID: 25565730; PMCID: PMC4184322

[8] Deutsch FT, Khoury SJ, Sunwoo JB, Elliott MS, Tran NT. Application of salivary noncoding microRNAs for the diagnosis of oral cancers. Head Neck. 2020 Oct;42(10):3072-3083. doi: 10.1002/hed.26348. Epub 2020 Jul 20. PMID: 32686879

[9] Shih YH, Wang TH, Shieh TM, Tseng YH. Oral Submucous Fibrosis: A Review on Etiopathogenesis, Diagnosis, and Therapy. Int J Mol Sci. 2019;20(12):2940. Published 2019 Jun 16. doi:10.3390/ijms20122940

[10] Passi D, Bhanot P, Kacker D, Chahal D, Atri M, Panwar Y. Oral submucous fibrosis: Newer proposed classification with critical updates in pathogenesis and management strategies.

Natl J Maxillofac Surg. 2017 Jul-Dec;8(2):89-94. doi: 10.4103/njms.NJMS_32_17. PMID: 29386809; PMCID: PMC5773997

[11] Gondivkar DSM, Gadbail DAR, Sarode DSC, et al. Treatment outcomes of laser therapy in oral submucous fibrosis-a systematic review. J Oral Biol Craniofac Res. 2020;10(3):253-258. doi: 10.1016/j.jobcr.2020.05.004

[12] Cuevas-Córdoba B, Santiago-García J. Saliva: a fluid of study for OMICS. OMICS. 2014 Feb;18(2):87-97. doi: 10.1089/omi.2013.0064. Epub 2014 Jan 3. PMID: 24404837

[13] Elashoff D, Zhou H, Reiss J, Wang J, Xiao H, Henson B, Hu S, Arellano M, Sinha U, Le A, Messadi D, Wang M, Nabili V, Lingen M, Morris D, Randolph T, Feng Z, Akin D, Kastratovic DA, Chia D, Abemayor E, Wong DT. Prevalidation of salivary biomarkers for oral cancer detection. Cancer Epidemiol Biomarkers Prev. 2012 Apr;21(4):664-72. doi: 10.1158/1055-9965.EPI-11-1093. Epub 2012 Feb 1. PMID: 22301830; PMCID: PMC3319329

[14] UMA MAHESWARI T, NIVEDHITHA M, RAMANI P. Expression profile of salivary micro-RNA-21 and 31 in oral potentially malignant disorders. Braz Oral Res. 2020;34. doi:10.1590/1807-3107bor-2020.vol34.0002

[15] Srivastava R, Jyoti B, Pradhan D, Siddiqui Z. Prevalence of oral submucous fibrosis in patients visiting dental OPD of a dental college in Kanpur: A demographic study. J Family Med Prim Care. 2019;8(8):2612-2617. Published 2019 Aug 28. doi: 10.4103/jfmpc.jfmpc_465_19.

[16] Memon AB, Rahman AAU, Channar KA, Zafar MS, Kumar N. Assessing the Quality of Life of Oral Submucous Fibrosis Patients: A Cross-Sectional Study Using the WHOQOL-BREF Tool. Int J Environ Res Public Health. 2021;18(18):9498. Published 2021 Sep 9. doi:10.3390/ijerph18189498.

[17] Gangane N, Grover S, Gupta A, Gupta S. P3.91. Reassessment of risk factors for oral cancer. Oral Oncology Supplement. 2009;3(1):231. doi: 10.1016/j.oos.2009.06.617

[18] Zini A, Nasser N, Vered Y. Oral and Pharyngeal Cancer Among the Arab Population in israel from 1970 to 2006. Asian Pacific Journal of Cancer Prevention. 2012;13(2):585-589. doi:10.7314/apjcp.2012.13.2.585

[19] Rao S, Mejia G, Roberts-Thomson K, Logan R. Epidemiology of Oral Cancer in Asia in the Past Decade- An Update (2000-2012). Asian Pacific Journal of Cancer Prevention. 2013;14(10):5567-5577. doi:10.7314/apjcp.2013.14.10.5567

[20] Madani A, Dikshit M, Bhaduri D, Jahromi A, Aghamolaei T. Relationship between Selected Socio-Demographic Factors and Cancer of Oral Cavity - A Case Control Study. Cancer Inform. 2010;9: CIN.S4774. doi:10.4137/cin. s4774

[21] Dalmay T. MicroRNAs and cancer. J Intern Med. 2008;263(4):366-375. doi:10.1111/j.1365-2796.2008. 01926.x

[22] Bartel D. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009;136(2):215-233. doi: 10.1016/j.cell.2009.01.002

[23] Liu C, Tsai M, Hung P et al. miR-31 Ablates Expression of the HIF Regulatory Factor FIH to Activate the HIF Pathway in Head and Neck Carcinoma. Cancer Res. 2010;70(4):1635-1644. doi: 10.1158/0008-5472.can-09-2291