MicroRNA, 2020, *9*, 295-302

RESEARCH ARTICLE

Expression of Salivary miRNA 21 in Oral Submucous Fibrosis (OSMF): An Observational Study

Shesha R. Prasad[1,*], Anuradha Pai[1], K. Shyamala[2] and Anisha Yaji[3]

[1]*Department of Oral Medicine and Radiology, The Oxford Dental College and Hospital, Bommanhalli, Hosur Road, Bangalore-560068, India;* [2]*Department of Oral and Maxillofacial Pathology, Raja Rajeswari Dental College & Hospital, Kumbalgodu, Bangalore - 560074, India;* [3]*Oral Medicine and Radiologist, Dental Department, Sri Krishna Sevashrama Hospital, Jaynagar, Bangalore - 560041, India*

Abstract: ***Objective:*** To observe the expression patterns of salivary mRNA 21 in different stages and grades of OSMF and also in habitual areca nut chewers without OSMF.

***Subjects and Methods*:** The study consisted of a total of 185 samples, where 61 patients had chewing habits (chewing gutkha and other forms of areca nut) and had OSMF (Group 1). 61 patients had chewing habits but did not have OSMF (Group 2), and 63 were normal healthy patients (control group) without any chewing habits (Group 3). Unstimulated saliva samples were collected from patients following the standard operating procedures. miRNA 21 was isolated and purified from saliva samples using the miRNeasy Mini Kit, Qiagen. The primers for miRNA relative quantification analysis were designed using the Primer Express software of Applied Biosystems. Quantification of all the samples was carried out using SYBR chemistry in an Applied Biosystems Real-Time PCR.

***Results*:** There was no statistically significant difference between the demographic characteristics of patients. There was a statistically significant difference between the expressions of miRNA 21 amongst the three groups noted in Kruskal Wallis test. (<0.001*) A post hoc test was perfomed to confirm the statistical difference between patients within all three groups. There was no statistically significant difference noted between the OSMF group and patients with chewing habits group (G1 *vs*. G2 p: 0.10), but there was a significant difference when compared with normal patients. (G1 *vs.* G3 p: <0.001*) and (G2 *vs.* G3 <0.001*)

***Conclusion*:** This study concludes that miRNA 21 is overexpressed in OSMF and chewing habit patients. But the expression levels were not significantly associated with the severity of the disease process. A long term and large scale studies are required to assess its application as a diagnostic profibrotic marker in OSMF.

ARTICLE HISTORY

Received: August 20, 2019
Revised: September 19, 2019
Accepted: December 09, 2019

DOI:
10.2174/2211536609666200127143749

Keywords: miRNA 21, OSMF, PMD, potentially malignant disorder, salivary miRNA, oral submucous fibrosis.

1. INTRODUCTION

Oral Submucous Fibrosis (OSMF) is a Potentially Malignant Disorder (PMD) of oral mucosa characterized by fibrosis of the subepithelial connective tissue. It has a reported prevalence ranging up to 0.4% and malignant transformation rate of 7.6% in the Indian rural population [1, 2]. As there is a lack of centralized registry to report, record, and follow up the OSMF patients, these ranges could be underestimated. This, in turn, could impact the worldwide disease rate as it is predominantly seen in Southeast Asian countries. The prediction of the malignant transformation of OSMF based on the primary clinical and histopathologic examinations is not reliable according to the literature [1]. Molecular markers like COX-2, type I plasminogen activator inhibitor, p53, keratinocyte growth factor-1, interleukin-6, *etc*., have been reviewed in literature as non-specific markers. A need for a specific marker that was up-regulated both in OSMF and Oral Squamous Cell Carcinoma (OSCC) resulted in exploring miRNA as a potential specific marker [3].

TGF-β superfamily comprising of TGF-β1, TGF-β2, TGF-β3, activins, and bone morphogenic proteins induces connective tissue growth factor stimulating Extracellular Matrix (ECM) synthesis. The presence of TGF-β1 is useful

*Address correspondence to this author at the Department of Oral Medicine and Radiology, The Oxford Dental College and Hospital, Bommanhalli, Hosur Road, Bangalore -560068, India; Tel/Fax: 9980056305;
E-mail: drsheshaprasad@gmail.com

2211-5374/20 $65.00+.00

in repair and regeneration of tissue, whereas its persistent presence can result in excessive fibrosis. The latter process has been implicated as being crucial in the development of OSMF, wherein TGF-β increases collagen production, causes cross-linking of mature collagen, which is resistant to lysis and inhibits degradation mechanisms [4].

The vital role of non-coding RNAs in physiology, development, metabolism, and disease has been emphasized for a long time. These RNAs are further classified as small interfering RNAs (siRNAs), microRNAs (miRNAs) and long ncRNAs (lncRNAs). The miRNAs are small double-stranded RNA comprising of 17-25 nucleotides, first identified as regulators of development in *Caenorhabditis elegans* larvae [4]. Until now [4-6], more than 1000 miRNA species are encoded by human cell, and they are capable of regulating the expression of hundreds of genes simultaneously [4-6]. These miRNAs are known to play a crucial role in cell differentiation, proliferation, apoptosis, and tumorigenesis through ancient regulatory mechanism evolved even before the divergence of single-celled organisms. The ongoing research has proved miRNA to be associated with oral cancer [5].

Amongst the human miRNAs, miRNA 21 is known to be up-regulated in fibrosis, cancer, and cardiovascular disease and has been studied extensively [6]. MiRNA 21 is established as an oncogenic miRNA and its overexpression was observed in oesophageal squamous cell carcinoma and prostate cancer [7].

Salivary miRNA 184 reportedly has higher sensitivity and specificity as a potential biomarker in PMDs, but miRNA 21 was chosen for this study. This was due to its profibrotic effects in initiating or fostering EMT. This is implied by its rapid inducibility after TGF- β treatment. TGF- β is known for its role in the induction of EMT and as a primary cytokine driving fibrosis in various organs, including the oral cavity [8, 9]. Also, miRNA 21 is a validated target of PTEN, as it is a negative regulator of PTEN, Akt/PKB activation. Both PTEN, Akt/PKB activation has been implicated in EMT. Post-transcriptional level [8] analysis of miRNA 21 showed that TGF- β and BMP4 (a member of TGF- superfamily) upregulate pre-miRNA 21 expression by 4-fold within 30 min after treatment while the expression of pri-miRNA 21 is unaltered [8]. Further experiments showed that elevated miRNA 21 levels were due to an increase in the Drosha processing of the pri-miRNA 21 transcript, which is mediated by SMAD proteins. After ligand stimulation, signal transducer SMADs (SMAD1/5 and SMAD2/3) were recruited to pri-miRNA 21 complex with a component of the Drosha microprocessor complex leading to fast processing of pri-miRNA 21 to pre-miRNA 21 and subsequent maturation. Interestingly, BMP6, which is also a member of the TGF- β superfamily, has been shown to inhibit miRNA 21 expression [8, 9].

Salivaomics is an emerging field wherein various biomarkers in saliva are identified and studied. Since the discovery of salivary miRNA, a lot of progress in identifying them to detect oral cancer is noted [10, 11].

Considering the link between miRNA 21, TGF- β, and OSMF, a study was designed to observe if salivary mRNA 21 is expressed in OSMF as well as patients exposed to its etiology.

2. METHODOLOGY

This observational cross-sectional study was conducted in the Oxford Dental College outpatient department. After obtaining the Institutional ethical clearance (Ref: 277/2013-14), a total of 185 samples were selected such that sixty-one patients had chewing habits (chewing gutkha and other forms of areca nut) and had OSMF (Group 1). Sixty-one patients had chewing habits but did not have OSMF (Group 2), and sixty- three were normal healthy patients (control group) without any chewing habits (Group 3). Patients who were clinically diagnosed as OSMF and the healthy controls with or without chewing habits who consented to participate in the study were included in the study.

The classification methods of Khanna *et al*., and Kerr *et al*., were used for diagnosing OSMF cases. Patients with systemic disorders like bleeding and clotting disorders, autoimmune disorders, or on drugs that can modify salivary composition were excluded from the study (Table **1**) [12, 13]. Apart from this, patients with any other chewing habits other than areca nut chewing were excluded.

Unstimulated saliva samples were collected from patients between 9 and 11 a.m. Patients were asked to refrain from eating, drinking, smoking, or using oral hygiene procedures for at least 1 hour prior to the collection of saliva. Patients were asked to rinse their mouth well with distilled water for 1 minute. Subjects were to either expectorate or swallow the water. Five minutes after the oral rinse, subjects were asked to spit 0.5-1.0 ml of saliva into a 5 ml sterile tube placed on ice. The saliva samples were stored in 3 ml "RNAlater® Solutions for RNA Stabilization and Storage by Life Technologies" at -20°C.

2.1. Extraction of RNA

A separate purification of large (mRNA) and small RNA (miRNA) using column-based technology was employed for OSMF and normal salivary RNA isolation. Approximately 3 ml of saliva was homogenized, and Large (>200 nt) and small RNA (<200 nt) fractions were isolated separately (RNeasy Plus mini kit and RNeasy MinElute cleanup kit, Qiagen) according to the supplementary Protocol. An Eppendorf 5417C microcentrifuge was used throughout the RNA extraction process. The RNeasy column was stored at 4°C for subsequent isolation of large RNA after extracting the small RNA according to the manufacturer's instructions. Later the samples were passed through a MinElute column by centrifuging at 12,000g for 21 seconds at 4°C. The same centrifugation conditions were used for two subsequent wash steps using the Qiagen RNeasy mini kit, and 500µL 80% ethanol, respectively.

The small RNA was eluted from the MinElute column in 20µL RNase-free water by centrifuging at 12,000g for 1 minute at 4°C. An optional DNase I treatment was performed using reagents from an RNase-free DNase set (Qiagen). Following DNase treatment, all 3 wash steps mentioned above

Table 1. Khanna and Andrade *et al*. and Kerr *et al.* classification.

Kerr *et al.* (2011) Classification		
Grade 1 – Mild Any features of the disease triad for OSMF (burning, depapillation, blanching or leathery mucosa) may be reported – and inter-incisal opening >35 mm		
Grade 2 – Moderate: Above features of OSMF + inter-incisal limitation of opening 20–35 mm		
Grade 3 – Severe: Above features of OSMF + inter-incisal opening <20 mm		
Grade 4A – OSMF + other potentially malignant disorder on clinical examination		
Grade 4B – OSMF with any grade of oral epithelial dysplasia on biopsy		
Grade 5 – OSMF + oral Squamous Cell Carcinoma (SCC)		
Khanna and Andrade *et al*. (1995) Classification		
Group I: Very early cases:	Common symptom is burning sensation in the mouth, acute ulceration and recurrent stomatitis and not associated with mouth opening limitation.	Fine fibrillar collagen network interspersed with marked edema, blood vessels dilated and congested, large aggregate of plump young fibroblasts present with abundant cytoplasm, inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils. The epithelium is normal
Group II: Early cases	Buccal mucosa appears mottled and marble like, widespread sheets of fibrosis palpable, interincisal distance of 26 to 35 mm.	Juxta-epithelial hyalinizalion present, collagen present as thickened but separate bundles, blood vessels dilated and congested, young fibroblasts seen in moderate number, inflammatory cells mainly consist of polymorphonuclear leukocytes with few eosinophils and occasional plasma cells, flattening or shortening of epithelial rete-pegs evident with varying degree of keratinization
Group III: Moderately advanced cases	Trismus, interincisal distance of 15 to 25 mm, buccal mucosa appears pale firmly attached to underlying tissues, atrophy of vermilion border, vertical fibrous bands palpable at the soft palate, pterygomandibular raphe and anterior faucial pillars.	Juxta-epithelial hyalinization present, thickened collagen bundles, residual edema, constricted blood vessels, mature fibroblasts with scanty cytoplasm and spindle-shaped nuclei, inflammatory exudates which consists of lymphocytes and plasma cells, epithelium markedly atrophic with loss of rete pegs, muscle fibers seen with thickened and dense collagen fibers.
Group IVA: Advanced cases	Severe trismus, interincisal distance of less than 15 mm, thickened faucial pillars, shrunken uvula, restricted tongue movement, presence of circular band around entire lip and mouth.	Collagen hyalinized smooth sheet, extensive fibrosis, obliterated the mucosal blood vessels, eliminated melanocytes, absent fibroblasts within the hyalinised zones, total loss of epithelial rete pegs, presence of mild to moderate atypia and extensive degeneration of muscle fibers.
Group IVB: Advanced cases	Presence of hyperkeratotic leukoplakia and/or squamous cell carcinoma	-

were repeated. The second of these steps had an increased centrifugation time of 2 minutes to dry the membrane.

The large RNA was eluted from the RNeasy column by applying 50µL RNase-free water to the membrane and centrifuging at 12,000g for 1 minute at 4°C. A portion of the purified large and small RNA was aliquoted for quantitative and qualitative analysis using a spectrophotometer. The remaining RNA was stored at -80°C for further use.

2.2. Analysis of miRNA Concentration

The miRNA concentration and purity were assessed using the spectrophotometer with a tightly controlled path length of 0.1cm. The RNA concentration was calculated using the formula:

RNA concentration (ng/µL) = (A260 x e)/b,

where: A260=Absorbance at 260nm, e=extinction coefficient (ng-cm/ml), b= pathlength(cm)

When analyzing small RNA samples, the extinction coefficient of 40 was manually entered, and RNA with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2 was deemed indicative of pure RNA. The presence of protein or phenol resulted in high absorption at 280nm, producing a lower A260/A280 ratio. A ratio at 260 and 230 nm (A260/A230) between 1.8 and 2.2 was also considered acceptable. Lower ratios indicated the carry-over of guanidinium salts.

The small-RNA enriched fractions were analyzed using the Small RNA Assay (Agilent Technologies), and the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples loaded onto the Agilent chip were separated by capillary electrophoresis according to their molecular weight. The intensity of fluorescence on each sample's electropherogram represented the amount of RNA of a given size. The small RNA assay was chosen for its high resolution in the 6-150 nucleotide range, allowing verification of small RNA retrieval and comparison of the small RNA component between tissue samples. The small RNA assay was carried out according to the Agilent small RNA kit guide. The electrodes were cleaned with RNase-free water for 5 minutes prior to use. For preparing the gel, the small RNA gel matrix

and small RNA dye concentrate were allowed to equilibrate to room temperature for 30 minutes; the latter reagent was protected from light throughout by covering the tube in tinfoil. The complete volume of gel will be spun at 10,000g for 15 minutes. The dye concentrate was vortexed for 10 seconds and briefly centrifuged. In a new 0.5 mL RNase-free tube, 2μL of dye concentrate and 40μL of the filtered gel was mixed thoroughly by careful pipetting. The gel/dye mix was then spun at 13,000g for 10 minutes, and samples were diluted to 1 ng/μL, within the quantitative and qualitative range of the assay. The RNA samples and RNA ladder were denatured at 70°C for 2 minutes and then placed on ice prior to use.

2.3. miRNA to cDNA Synthesis

Small RNA (5 ng) was reverse-transcribed using MultiScribe Reverse Transcriptase (AppliedBiosystems), and each reaction was primed using a miRNA-specific stem-loop primer. Where sequences are available, primers were obtained from life technologies. Otherwise, assays containing the RT stem-loop primer and the PCR primers and probes were used (Applied Biosystems).

Samples obtained were further incubated at 16°C for 30 minutes, 42°C for 30 minutes and finally 70°C for 15 minutes to denature double-stranded duplexes. The reaction was performed using a thermal cycler (Applied Biosystems). An nRT-negative control was included in each batch of the reactions. At the same time, to generate the cDNA template for the endogenous control PCR reactions, the first-strand cDNA was synthesized using 1μg of RNA from the same samples for stem-loop reverse transcription, and oligo(dT) as the primer. The reaction parameters were incubation at 42°C for 30 min, 70°C for 15 min and then kept at 4°C.

2.4. Primer Sequences

Stem-loop RT Primer:

5-'CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGTCAACATC-3'.

2.5. Real-Time PCR Study

Real-time PCR was performed with an Applied Biosystems Step One detection system in a 15 μl reaction volume. All reactions were done in triplicate. For the endogenous control, GAPDH and 1 μl of cDNA synthesized by using oligo(dT) were used as the template.

The reaction parameters were incubated at 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min were performed. For expression analysis, the experiment was designed to use the matched normal sample as the control, so the relative quantification of miRNA 21 in OSMF and chewing habit group samples were calculated using the equation:

Amount of target=2-ΔΔCt (17), ΔΔC t= (CtmiRNA 21 - CtGAPDH) OSMF- (CtmiRNA 21 - CtGAPDH) matched normal sample.

For the matched normal tissue control sample, ΔΔCt is zero, and 2-ΔΔCt is 1. Melting curves were generated for each real-time PCR to verify the amplification of only the desired product. Considering the expression of miRNA 21 in Normal sample (N) as 1, the expression of all the samples was calculated.

Later, the miRNA of the N group was quantified by comparing it to the small nuclear RNA C/D Box 38B (SNORD38B) as standard.

3. STATISTICAL ANALYSIS

Statistical Package for Social Sciences [SPSS] for Windows Version 22.0 Released 2013. Armonk, NY: IBM Corp. was used to perform statistical analyses. Descriptive analysis of all the explanatory and outcome parameters will be performed using mean and standard deviation for quantitative variables, frequency, and proportions for categorical variables. Chi-square test, Kruskal Wallis test followed by Mann Whitney U test was used to compare the mean miRNA expression folds between different groups, different grades of OSMF as per Kerr *et al.* grading system and also between different stages of OSMF as per Khanna & Andrade *et al*., staging system.

Spearman's correlation test was used to estimate the relationship between the expression of miRNA folds, chewing habit frequency, Duration & Mouth opening among OSMF and chewing habits group.

The level of significance was set at $P<0.05$.

4. RESULTS

This study consisted of 185 samples, out of which 61 were patients with OSMF (Group1), 61 patients with chewing habit without any disease (Group 2), and 63 were normal healthy patients (Group 3).

The demographic characteristics of the patients were comparable among the groups (Figs. **1** and **2**). Males were more than females in all three groups (Group 1: M=47, F=14; Group 2: M=46 F=15; Group 3: M=47, F=16). The mean age for all three groups was 34.1+/- 13.4, 37.3+/- 17.1 and 32.8+/- 11 for group 1, group 2, and group 3, respectively.

Spearman's correlation test revealed negatively very weak correlation between miRNA 21 expression folds and duration and frequency of chewing habits in OSMF patients and mouth opening in patients with chewing habits (Table **2**). A very weak correlation was noted between expression folds and mouth opening in OSMF patients (Group 1) and the duration and frequency of chewing habit in patients with chewing habit (Group 2).

Mean miRNA 21 expressions between the groups were compared using Kruskal Wallis Test (Table **3**). There was a statistically significant difference between the three groups. Mann Whitney U post hoc test was conducted for multiple comparisons of mean differences between different groups (Table **4**). There was no significant difference between the OSMF and chewing habit groups. But, the normal group (Group 3) was significantly different from both the OSMF (Group 1) and the chewing habit (Group 2) group.

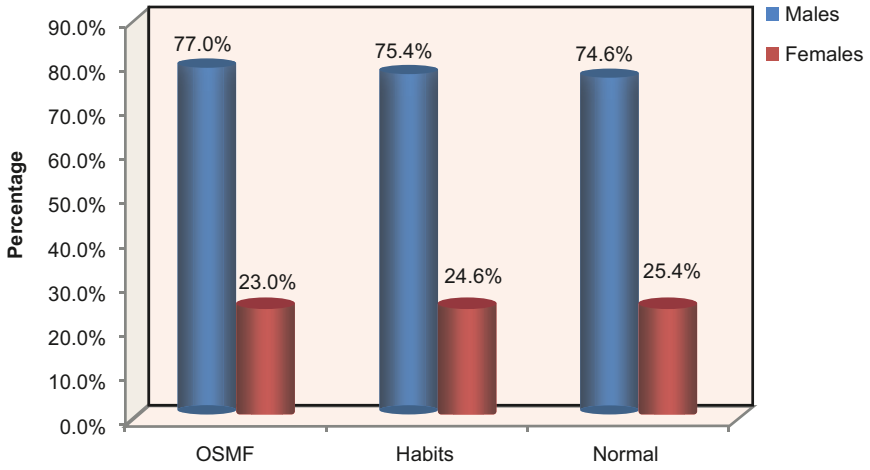

Fig. (1). Gender-wise distribution among different study groups.

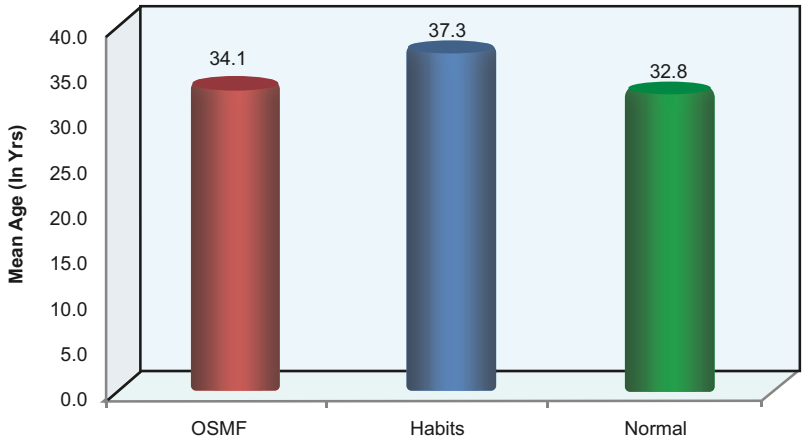

Fig. (2). Age-wise distribution among different study groups.

Table 2. Correlation between expression of miRNA folds, habit frequency, duration & Mouth Opening (MO) among different study groups.

Variables	Values	Duration	Frequency	Mouth Opening (MO)
Expression of folds	rho	-0.05	-0.21	0.04
	P-Value	0.70	0.10	0.78
	N	61	61	61
Expression of folds	rho	0.17	0.04	-0.11
	P-Value	0.20	0.74	0.39
	N	61	61	61

Table 3. Comparison of mean miRNA expression folds between different groups using Kruskal Wallis test.

Groups	N	Mean	SD	Min	Max	H	P-Value
OSMF (Group 1)	61	14.31	23.33	0	123.0	-	-
Habits (Group 2)	61	6.61	14.65	0	74.8	54.074	<0.001*
Normal (Group 3)	63	0.50	0.30	0	1.0	-	-

Table 4. Multiple comparison of mean differences between different groups using Mann Whitney U post hoc test.

Groups	G1 *Vs.* G2	G1 *Vs.* G3	G2 *Vs.* G3
P-Value	0.10	<0.001*	<0.001*

Table 5. Comparison of mean miRNA expression fold change between different grades of OSMF as per Kerr *et al.* grading system and Khanna & Andrade *et al.* staging system using Kruskal Wallis test.

Grading	N	Mean	SD	Min	Max	H	P-Value
Grade 1	3	5.34	4.39	1.9	10.3	-	-
Grade 2	51	15.01	24.99	0.0	123.0	-	-
Grade 3	3	4.45	2.89	1.5	7.3	-	-
Grade 4	1	2.23	.	2.2	2.2	3.302	0.65
Grade 4B	2	32.54	4.04	29.7	35.4	-	-
Grade 5	1	11.04	.	11.0	11.0	-	-

Table 6. Comparison of mean miRNA expression fold change between different grades of OSMF as per Khanna & Andrade *et al.* staging system using Kruskal Wallis test.

Staging	N	Mean	SD	Min	Max	H	P-Value
Stage 1	3	28.75	35.27	6.5	69.4	-	-
Stage 2	28	10.24	19.84	0.0	92.8	6.781	0.08
Stage 3	24	14.84	26.36	0.0	123.0	-	-
Stage 4	6	24.00	19.51	1.1	46.7	-	-

Comparison of mean miRNA 21 expression folds between different stages of OSMF as per Kerr *et al.* and Khanna & Andrade *et al.* staging system using the Kruskal Wallis test revealed there was no significant difference between the OSMF stages and miRNA 21 expression (Tables **5** and **6**).

5. DISCUSSION

MiRNA is an established key molecular component of cancer biology right from tumor growth to immune invasion. MiRNAs ability to act as oncogenes or tumor suppressor genes in several cell-signaling pathways essential to carcinogenesis resulted in its use in targeted cancer studies. Their remarkable stability and resistance to degradation make them excellent candidates for cancer biomarkers. Shah *et al.*, have reviewed the role of miRNA as a cancer biomarker and reported its role in diagnosis, prognosis as well as a therapeutic biomarker [14, 15]. Their association with several other with or without dysplasia has also been assessed, including Oral Lichen Planus, Oral Verrucous Leukoplakia and Recurrent Aphthous Stomatitis [16].

Multiple miRNAs were assessed in relation to oral PMDs including miRNA 21, 27b, 145, 181, 181b and 345. Cervigne *et al*., reported that over-expression of miRNA 21, miRNA 181b and miRNA 345 may play an important role in the malignant transformation of leukoplakia [16-18]. Various studies have proved miRNA 27b, miRNA 145, miRNA 181, and miRNA 21 had statistically significant sensitivity and specificity to detect early malignancy [17, 18]. Hung *et al*., assessed miRNA 31 and miRNA 21 in saliva samples of oral PMD reported them to be a useful tool in screening high-risk patient especially miRNA 31 [18]. In another study miRNA 21, miRNA 184, and miRNA145 were assessed in oral PMDs [19]. There was a highly significant increase in salivary miRNA 21 and miRNA 184 in oral SCC and PMD (with and without dysplasia) when compared to healthy and disease controls. To the best of our knowledge, there is only one study that has assessed serum miRNA 21 in OSMF [20]. Expression deregulation of miRNA 204, miRNA 31, miRNA 133a, miRNA 7 and miRNA 206 and their possible target genes in 23 cancers, 18 Leukoplakia, 12 Lichen Planus, and 23 OSMF tissues compared to 20 healthy tissues was determined by qPCR method and they reported up-regulation of genes in all except OSMF which was downregulated. A recent systematic review also noted that there was a lack of studies that assessed miRNA levels in OSMF. Hence, in this study, miRNA levels in OSMF cases along with normal and chewing habit cases were considered [8, 21].

Saliva is one of the 12 biologically sources of miRNA apart from amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, plasma, pleural fluid, seminal fluid, tears, urine and saliva [21]. Hung *et al.* in 2016 compared saliva samples with tissue samples and concluded that saliva samples were significantly better for predicting malignancy than tissue samples. In our study, saliva samples were taken to assess miRNA levels [22].

The study consisted of 185 samples with comparable demographic characteristics amongst all three groups. (Figs. **1** and **2**) From all these patients, the samples were collected under standard operating procedures eliminating the risk of sample contamination. Normalization is important for accurate quantification of RNA levels using reverse transcription-PCR (qRT-PCR). The most commonly used normalization strategies for expression of circulating miRNAs are Global Mean Expression, exogenous (spiked-in) miRNAs, and endogenous controls. The most widely used method is by endogenous controls such as miRNA, small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA). In order to reduce the technical variation and avoid the misinterpretation of the data, a suitable reference gene is used based on other studies or searching stable miRNAs in their own samples [22]. As there is no consensus on which miRNA is best for normalization in our study, both exogenous and endogenous controls were used. For endogenous control, the first sample from group 3 was considered normal, and an increase in miRNA 21 folds with respect to that samples were calculated.

A comparison of mean miRNA 21 expression folds between different groups using Kruskal Wallis revealed a statistically significant difference between 3 groups (Table **3**). This was in agreement with Singh *et al*. study assessed miRNA 21in serum of OSMF patients, whereas in our study, saliva was used as a source of the sample. To the best of our knowledge, this was the only study to assess miRNA 21 in the saliva of OSMF patients [20].

Multiple comparisons of mean differences between different groups using Mann Whitney U post hoc test revealed that there was an increase in miRNA expression folds in both OSMF and chewing habit groups when compared with normal patients group. This has not been assessed in any of the studies previously. This also points out the recent trend of tissue changes post-exposure to chewing habits despite the absence of disease [2].

When the miRNA 21 expression amongst the disease group (Group 1) was compared according to its clinical and histological grading, there was no statistically significant difference between the groups. Singh *et al*. study the significant high expression of miRNA 21 was observed from stages I-IV and were correlated with different stages from early to a later stage [21]. The difference between the source of the sample (*i.e.* saliva and serum) could be proposed as one of the reasons for this variation.

6. CLINICAL TRANSLATION OF THE STUDY FINDINGS

As we could see from the study results, miRNA 21 is elevated in both patients who had OSMF and who didn't, but had a chewing habit. This opens an array of possibilities that could best explain the development of disease even after the chewing habit cessation or clinically mild cases progressing to malignancy.

Zahran *et al*. assessed miRNA 21 in SCC and PMD, showing dysplastic changes reported lack of significant difference between both. They suggested that the aberrant expression of miRNA 21 in saliva might reflect an early molecular event in the pathogenesis of OSCC. They proposed that a 4- to 4.5-fold increase in miRNA 21 as a reasonable cutoff point for frank cancer transformation with a specificity of 65%, sensitivity 65% and specificity 90%, sensitivity 60%, respectively [18]. In our study, 30/61, OSMF cases, and 13/61 patients with chewing habit and without OSMF cases had more than fourfold rise in miRNA 21, whereas none of the normal cases had more than one fold rise in miRNA 21.

It was noted that two out of 61 cases of OSMF who had more than 4.5 fold increase in miRNA 21 presented with SCC (squamous cell carcinoma) of buccal mucosa over a span of 1 and a half years from the time of assessment. We could not follow up on all the cases (Group 1 and Group 2) that showed increased miRNA 21 expression. Therefore, a long term follows up the study of patients with a more than four-fold increase in miRNA 21 can shed value on its application as an indicator of malignant transformation.

7. LIMITATIONS OF THE STUDY

The lack of statistical significance difference of miRNA 21 between the OSMF group and the disease-free chewing group indicate that further studies are necessary, and do not exclude that differences among groups of miRNA 21 levels may be artefactual. Additional tissue or cell studies showing a causative role of miRNA 21 in oral fibrosis or the development of oral neoplasia are required to validate the herein presented hypothesis.

CONCLUSION

This study observed a definite increase in miRNA 21 expression in OSMF cases compared to normal cases without any areca nut chewing habit. This finding is also consistent in patients who had a chewing habit and no disease. A possible explanation could be molecular changes occurring in the oral tissues much before the clinical or histologic manifestation of the disease. Hence, miRNA 21 could be a risk indicator for OSMF development, as well as progression to oral cancer as it is an oncogenic gene. Future studies with a long term follow up protocol in such patients is necessary to ascertain the above conclusions.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was taken from the Institutional Review Board of The Oxford Dental College, Bangalore, India. (Ref NO: 277/2013/2014).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. The reported experiments in accordance with the ethical standards of the com-

mittee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written informed consent was taken from all participants.

AVAILABILITY OF DATA AND MATERIALS

Data sharing is not applicable to this article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Ali FM, Aher V, Prasant MC, Bhushan P, Mudhol A, Suryavanshi H. Oral submucous fibrosis: Comparing clinical grading with duration and frequency of habit among areca nut and its products chewers. J Cancer Res Ther 2013; 9(3): 471-6. http://dx.doi.org/10.4103/0973-1482.119353 PMID: 24125985

[2] Poonja PA, Sattur AP, Burde KN, Hiremath SV. A study on genetic polymorphism in matrix metalloproteinases-3 in oral submucous fibrosis patients and healthy individuals. App Cancer Res 2017; 37: 4. http://dx.doi.org/10.1186/s41241-016-0009-1

[3] Liao P-H, Yang H-W, Huang Y-F. Genetic expression signatures of oral submucous fibrosis and oral cancer-A preliminary microarray report. J Dent Sci 2016; 11(4): 457-62. http://dx.doi.org/10.1016/j.jds.2013.02.017 PMID: 30895012

[4] Rajalalitha P, Vali S. Molecular pathogenesis of oral submucous fibrosis – A collagen metabolic disorder. J Oral Pathol Med 2005; 34: 321-8.

[5] Perez P, Jang SI, Alevizos I. Emerging landscape of non-coding RNAs in oral health and disease. Oral Dis 2014; 20(3): 226-35. http://dx.doi.org/10.1111/odi.12142 PMID: 23781896

[6] Osada H, Takahashi T. MicroRNAs in biological processes and carcinogenesis. Carcinogenesis 2007; 28(1): 2-12. http://dx.doi.org/10.1093/carcin/bgl185 PMID: 17028302

[7] Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004; 116(2): 281-97. http://dx.doi.org/10.1016/S0092-8674(04)00045-5 PMID: 14744438

[8] 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. http://dx.doi.org/10.1038/srep32735 PMID: 27597234

[9] Maheswari TNU, Venugopal A, Sureshbabu NM, Ramani P. Salivary micro RNA as a potential biomarker in oral potentially malignant disorders: A systematic review. Ci Ji Yi Xue Za Zhi 2018; 30(2): 55-60. http://dx.doi.org/10.4103/tcmj.tcmj_114_17 PMID: 29875583

[10] Cai Y, Yu X, Hu S, Yu J. A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics 2009; 7(4): 147-54. http://dx.doi.org/10.1016/S1672-0229(08)60044-3 PMID: 20172487

[11] Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. RNA Biol 2011; 8(5): 706-13. http://dx.doi.org/10.4161/rna.8.5.16154 PMID: 21712654

[12] Ranganathan K, Mishra G. An overview of classification schemes for oral submucous fibrosis. J Oral Maxillofac Pathol 2006; 10(2): 55-9.

[13] Kerr AR, Warnakulasuriya S, Mighell AJ, *et al.* A systematic review of medical interventions for oral submucous fibrosis and future research opportunities. Oral Dis 2011; 17(Suppl. 1): 42-57. http://dx.doi.org/10.1111/j.1601-0825.2011.01791.x PMID: 21382138

[14] Rapado-González Ó, Majem B, Muinelo-Romay L, *et al.* Human salivary microRNAs in cancer. J Cancer 2018; 9(4): 638-49. http://dx.doi.org/10.7150/jca.21180 PMID: 29556321

[15] Shah S, Jadhav K, Shah V, Gupta N, Dagrus K. miRNA 21: Diagnostic prognostic and therapeutic marker for oral cancer. MicroRNA 2016; 5(3): 175-9. http://dx.doi.org/10.2174/2211536605666160919115323 PMID: 27739364

[16] Cervigne NK, Reis PP, Machado J, *et al.* Identification of a microRNA signature associated with progression of leukoplakia to oral carcinoma. Hum Mol Genet 2009; 18(24): 4818-29. http://dx.doi.org/10.1093/hmg/ddp446 PMID: 19776030

[17] Momen-Heravi F, Trachtenberg AJ, Kuo WP, Cheng YS. Genomewide study of salivary microRNAs for detection of oral cancer. J Dent Res 2014; 93(7) (Suppl.): 86S-93S. http://dx.doi.org/10.1177/0022034514531018 PMID: 24718111

[18] Zahran F, Ghalwash D, Shaker O, Al-Johani K, Scully C. Salivary microRNAs in oral cancer. Oral Dis 2015; 21(6): 739-47. http://dx.doi.org/10.1111/odi.12340 PMID: 25784212

[19] Hung KF, Liu CJ, Chiu PC, *et al.* MicroRNA-31 upregulation predicts increased risk of progression of oral potentially malignant disorder. Oral Oncol 2016; 53: 42-7. http://dx.doi.org/10.1016/j.oraloncology.2015.11.017 PMID: 26675284

[20] Singh P, Srivastava AN, Sharma R, *et al.* Circulating microRNA-21 expression as a novel serum biomarker for oral sub-mucous fibrosis and oral squamous cell carcinoma. Asian Pac J Cancer Prev 2018; 19(4): 1053-7. PMID: 29699056

[21] Marabita F, de Candia P, Torri A, Tegnér J, Abrignani S, Rossi RL. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. Brief Bioinform 2016; 17(2): 204-12. http://dx.doi.org/10.1093/bib/bbv056 PMID: 26238539

[22] Hedbäck N, Jensen DH, Specht L, *et al.* MiR-21 expression in the tumor stroma of oral squamous cell carcinoma: An independent biomarker of disease free survival. PLoS One 2014; 9(4)e95193 http://dx.doi.org/10.1371/journal.pone.0095193 PMID: 24755828