Original Article

Evaluation of Novel MicroRNA Profile-21 and 191 in Oral Leukoplakia and Oral Squamous Cell Carcinoma in Comparison with Healthy Tissues – A Cross-Sectional Study

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Abstract

Objective: The objective of this study was to identify whether microRNA (miR)-21 and miR-191 could be used as a potential biomarker in patients diagnosed with oral leukoplakia (OL) and oral squamous cell carcinoma. The aim of this study was to evaluate the expression of miR-21 and miR-191 in patients diagnosed with OL and oral squamous cell carcinoma. **Materials and Methods:** Fifteen patients each diagnosed with OL and oral squamous cell carcinoma and 15 healthy controls were recruited for the study. The miR was extracted from the tissue samples for the evaluation of miR-21 and miR-191 using RT-qPCR. **Results:** The miR-21 (P = 0.0005) and miR-191 (P = 0.0094) were significantly expressed in oral squamous cell carcinoma in comparison with healthy tissues. The expression of miR-191 was also significantly overexpressed (P = 0.0460) in OL in comparison with healthy tissues. **Conclusion:** The profile miR-21 and miR-191 could be used as a novel biomarker for oral squamous cell carcinoma and miR-191 could be used as a biomarker for OL.

Keywords: *MicroRNA, molecular biology, oral leukoplakia, oral squamous cell carcinoma, premalignant*

Introduction

Oral squamous cell carcinoma accounts for the eighth most common cancer worldwide, with an incidence of 354,864 reported cases and around 177,384 deaths annually. The incidence rate is higher in India, Sri Lanka, and Papua New Guinea.[1] They constitute about 30%–40% of cancers in India.[2] Carcinogenesis is a multistep pathway, which is influenced by local and molecular factors along with genetic susceptibility and plays an essential role in the progression of the disease.[3] The chronic exposure to local factors in oral cavity such as tobacco, areca nut, pan, and trauma to the exposed site facilitates to induce changes at cellular level, where there is stepwise transition of normal cells to malignant cells.[4] The process favors the transition of oral premalignant lesion into oral cancer.

The oral premalignant lesions have higher chance for malignant transformation and they are defined as "a morphologically altered tissue in which oral cancer is more likely to occur than its apparently normal counterpart."[5] The oral premalignant lesions are oral leukoplakia (OL), erythroplakia, and lesions due to reverse smoking in the palate.[6] OL is the most common premalignant lesion of the oral cavity, and the major etiological factors are consumption of smoking and smokeless tobacco, alcohol, and nutritional deficiencies. The center of attention to OL is due to its higher global incidence rate of 1.49%–2.6% in males, and its malignant transformation rate which accounts for 0.13%–17.9% of total reported cases.[7,8]

Evolution of cancer is not induced by a single-gene mutation. The malignant transformation is associated with a dysregulation in cellular and molecular biology. There have been various attempts to study and understand the gene pathway in malignant transformation of a normal cell. The malignant transformation is associated with the alteration at the cellular level through the signaling pathways induced by microRNAs (miRs).[9]

In the recent decade, there has been an increased interest toward the small

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nonprotein coding gene regulators, which is widely present in humans as miRs. The miRs were discovered from *Caenorhabditis elegans* in 1993. They are small, noncoding RNA molecules with 20–25 nucleotides in length. They have a major role as regulators of homeostasis at the cellular level as they monitor and control the cellular processes such as morphogenesis, differentiation, proliferation, apoptosis, and survival mechanisms.[10]

In carcinogenesis, miRs plays an essential role in cellular differentiation and they hold a significant phenotypic signature for every individual cancer by varying from specific tissue to another tissue invariable to individuals. They are widely studied to understand the cancer pathway. There are two types of miRs; they are oncogenic and tumor suppressors. Oncogenic miRs promote carcinogenesis while prevention is done by tumor suppressor genes. These play an essential role in the signaling pathway of carcinogenesis.[11]

The miR-21 is an oncogenic miR, and it has been significantly upregulated in oral squamous cell carcinoma and premalignant lesions.[10,11] The miR-191 is significantly expressed in human cancers of breast, prostate, colon, and oral cavity.[12] The dysregulation of miR-191 has been reported in oral cancer, and its expression in OL is yet to be explored. Literature survey reveals a paucity of research in assessing its expression in OL.

Hence, the study was undertaken with an aim to emphasize the importance of initial stages of screening and evaluate oral potentially malignant disorders and to prevent their malignant transformation. The current study was conducted with the aim to evaluate the expression of miR-191 in OL and oral squamous cell carcinoma in comparison with healthy tissues.

Materials and Methods

Study design

This was a cross-sectional study.

Study protocol

The study was carried out in the Outpatient Department of Oral Medicine and Radiology, SRM Dental College. The study was approved by the Institutional Review Board and Ethical Committee (SRMDC/IRB/2018/MDS/No. 903 dated December 11, 2018). The study was conducted over a period of 2 years from December 2018 to October 2020. The study protocol followed all the recommendations of Helsinki Declaration (2013).

Sample size estimation

The sample size was calculated using G*Power software; the total sample size for the study was 45. The study has three groups: Group I: OL (n = 15), Group II: oral squamous cell carcinoma (n = 15), and Group III: healthy tissues (n = 15).

Subject selection

The study included individuals aged from 40 to 70 years of age. The patients clinically diagnosed with OL and oral squamous cell carcinoma and who had a habit of smoking and chewing tobacco were selected for Group I and Group II, respectively. According to the clinical diagnostic criteria for OL by the World Health Organization (WHO) 1980 – "Homogeneous Leukoplakia" – lesion which is uniformly white and scrapable was included in Group I of the study. The clinical diagnostic criteria for oral squamous cell carcinoma by the American Joint Committee on Cancer (AJCC), 2010-TNM Stage I–III was included in Group II. They were recruited in the study after obtaining informed consent according to the Institutional Review and Ethical Board of the college. Group III was healthy individuals who were willing to participate in the study, and they were recruited as controls with negative health examination including detailed history and oral examination and along with informed consent. Patients with any other oral premalignant lesion or condition, history of treated or untreated malignancies of any other system, patients who were contraindicated for biopsy, and who were not willing to participate in the study were excluded from the study.

Sample collection

Tissue biopsies were performed for Group I [Figure 1] and Group II [Figure 2] patients with increased caution for excessive bleeding and other complications. Tobacco counseling was given for the patients during their first visit, and reinforcement was given after the biopsy. The obtained tissue sample was divided into two parts; one part was stored in 10% of formaldehyde for histopathological examination and the other part was stored in the aliquots with "RNA later" reagent. The tissue samples from healthy individuals were collected from the extraction site and stored in separate aliquots. The aliquots were stored at 4°C for 48 h and then at −20°C until further process.

Extraction of messenger RNA, complementary DNA, and microRNA

The tissue samples were subjected to NucleoSpin® RNA kit (cat#740955.50, Macherey-Nagel, Germany) for extraction of messenger RNA (mRNA), as this kit is capable in the enrichment of the extraction of RNA along with smaller molecules like miR by detaching the genomic DNA from the tissue. The tissue sample was homogenized and was subjected to lysis with 350 µl of lysis buffer supplemented with 80 µl of protease (Mat#1016330, Qiagen, Germany). The disruption of the cellular and nuclear membrane occurs in the procedure, which release both total RNA and miR species, and to remove the undisrupted tissue, the samples were subjected to centrifuge at room temperature for 3 min. The precipitation of total RNA and miR fractions was performed in vortex for 30 s by adding equal volume

Figure 1: The figure reveals Homogeneous leukoplakia in the left buccal mucosa

Figure 2: The figure reveals Stage II Oral Cancer in the right buccal mucosa

of 70% ethanol to regulate the RNA binding conditions. The silica membrane technology is used to capture the RNA particles; therefore, the acquired sample was subjected to RNA capture columns with charged silica membrane. The total RNA and miR bounded to the silica membrane and the other molecules were discarded. The obtained RNA molecules were washed with buffers to remove the residual DNA and protein molecules. The silica membrane was dried by centrifuging at 12,000 rpm for 3 min, and 20 µl of DNase/RNase-free water was added to the bounded RNA molecules. The sample was centrifuged at 12,000 rpm for 1 min at room temperature, and the eluate was collected. The total RNA and miR molecule quantification was performed using Qubit™ RNA BR Assay Kit. The mRNA transcription was done to synthesize cDNA and to transcribe both mRNA and miR molecules using miScript kit.

Establishment of standards for quantitative real-time PCR

To quantitatively determine the copy numbers of miR molecules (relative to each other and among the samples), a linear graph with serial dilutions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene products of PCR was established. The miR-21[13] and miR-191[14] and GAPDH molecules were amplified with similar efficiency.

The copy number of PCR amplicons present in nanograms of GAPDH gel eluate was established by using the following formula:

$$\frac{(\text{ng}/\mu\text{L})\, x\, 6.022\times10^{23}}{(\text{Length of amplicon in base pair})\times1\times10^{9}\times650}$$

As the copy numbers were determined, GAPDH serial dilutions eluate was made and it was obtained concentration from 1 × 106 to 1 × 101. These diluted samples were analyzed by Real Time-Polymerase Chain Reaction (RT-PCR) in the presence of QuantiNova SYBR Green PCR Kit (Cat#208052, Qiagen, Germany) Qiagen 5-plex rotor gene in RT-PCR to establish a linear standard graph.

Real-time polymerase chain reaction

The primers were outlined in a way to selectively amplify only miR-21 and miR-191 cDNA during RT-PCR. The miR amplifications from the samples were performed in a 20-µl reaction with Type IT high-resolution melting analysis kit (HRM). It was further subjected to denaturation for 4 min at 95°C, and the amplification was used to quantitative analyze both the miR samples. The miR concentration in each specimen was denoted as copies/µL. The copy numbers relate to both presence and to estimate the quantification of miR molecules. For example, when a sample contains higher concentration of miR-21 molecules, reverse transcription will produce higher amount of miR-21 cDNA and vice versa. These samples when analyzed by real-time PCR will show a higher copy number of miR-21 molecules.

Statistical analysis

The expression levels of miRs were compared between three groups using Kruskal–Wallis test with Dunn's *post hoc* analysis. The correlation between histopathological grading and miRs was analyzed using Pearson's correlation. Receiver operating curve curves were established to evaluate the prognostic value of miR in differentiating tissues. $P < 0.05$ was considered statistically significant with 95% confidence interval. All the statistical analysis was performed with STATA/IC version 16.1 statistical software (STATA Corp., College Station, Texas, USA).

Results

qPCR tissue samples

Total RNA and miR extracted from the tissue samples were first quantified with Qubit fluorometer. A 100 ng of total RNA and miR from each sample was subjected to

reverse transcription for synthesizing cDNA. As cDNA is synthesized, the miR-specific primers were utilized to amplify respective miR molecules present in the tissue samples by real-time PCR. The analysis showed miR-21 and miR-191, the specific amplification curves with different cycle threshold values.

To determine the copy numbers of each miR molecule, the amplification curve [Figures 3 and 4] was normalized to establish a cutoff threshold value in reference to the linear standard graph. The standards were run every time along with miR-21 and miR-191 specimen sample. The amplification curve and linear standard graph of each micro RNA molecule was performed by Internal software (Qiagen, Germany). To confirm the specificity of amplifications of miR-21 and miR-191, the tissue samples were subjected to melt curve analysis [Figures 5 and 6] at the end of each run. The specificity of the amplification was confirmed by a single positive peak for miR-21 and miR-191 samples.

To determine the relative expression of miR-21 and miR-191 in tissue samples, the copy numbers of miR-21 and miR-191 molecules present in each sample were pooled into three groups, Group I: OL, Group II: oral squamous cell carcinoma, and Group III: healthy tissue. The copy numbers of samples within each group were then processed to get a relative expression pattern of miRs in each tissue sample, and it was assessed statistically according to tissue type, gender, and site.

Kruskal–Wallis test compared the expression of miR21 and miR191 between three tissues [Table 1], and there was a significant (P = 0.0007 and P = 0.0169) difference among the three groups, respectively.

The Dunn's *post hoc* analysis [Table 2] revealed that there was a significant difference in the expression levels of miR-21 and miR-191 in between oral squamous cell carcinoma (OSCC) and healthy tissue, P = 0.0005 and P = 0.0094, respectively. The expression of miR-21 was higher in OSCC compared to OL, P = 0.0059. The expression of miR-191 was significantly higher in OL when compared to normal tissue, P = 0.0460.

In OL, the correlation between histopathological staging and miR expression was assessed using Pearson's correlation coefficient "*r*" [Table 3]. The correlation between H/P staging and miR-21 was − 0.2764 and for miR-191 was − 0.2543 indicating a weak negative correlation. The correlation between miR-21 and miR-191 in assessing OL was 0.5587 indicating a moderate correlation.

According to the results, the expression of miR-21 and miR-191 was found to be higher in oral squamous cell carcinoma samples in comparison with OL and normal tissue, and miR-191 expression was found to be higher in OL in comparison with normal tissues.

Discussion

OL is a term derived from two distinctive Greek words, "Leucos" mean white and "Plakia" means patch. The term was coined by Ernő Schwimmer, a Hungarian dermatologist in 1877. It is defined by Saman Warnakulasuriya *et al*. in the year 2007 as "Oral leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer." In India, the annual incidence rate is around 1.1–2.4/1000/year in men and 0.2–1.3/1000/year in women. It is more predominant in males compared to females with a male: female ratio of 3.2:1.[15] It occurs mostly in the 4th–7th decades of life. It can arise in any part of the oral mucosa, and the most common occurrence sites are buccal mucosa (21.9%–46%), mandibular alveolar region (25.2%–40%), hard palate (27%), tongue (26%), and floor of the mouth (19.3%).[16] The favoring feature of OL for malignant transformation is the presence of cellular dysplasia and molecular events. There is a higher chance for OL to undergo malignant transformation.

Oral cancer incidence rate is higher in tobacco consumers. In India, there is a wide range of custom for smoking tobacco and chewing smokeless tobacco. Tobacco smokers are 27 times at higher risk to develop oral cancer than nonsmokers. They have a 5-year survival rate with 55%–60% for patients diagnosed at early stage of cancer, while at the advanced stage, it is decreased to 30%–40%.[17]

The histopathological evaluation reveals the changes at cellular level. The molecular changes include the epigenetic and genetic alterations in the mucosal cells due to both intrinsic and extrinsic factors.[18] To enhance the treatment strategies and survival rate for the patients, it is essential to understand the cancer biology. Molecular biomarkers provide an in-depth insight on cancer biology.

The first evidence of miR expression in cancer was reported by Dr. Carlo M Croce. There are more than 1000 miRs present in the human genome.[19] The biogenesis of miR is the conversion of primary miR by RNA polymerase II enzyme to pre miR by binding to the 3'-untranslated region of mRNA. The pre miR is of 60–70 nucleotides in length, which is transported to the cytoplasm for the conversion of pre miR to a short stranded miR by RNase III endonucleases in the cytoplasm.[19] The miRs bind at multiple sites of mRNA and regulate expression at post transcriptional level. They hold a significant phenotypic signature for every individual cancer by varying from specific tissue to another tissue invariable to individuals.[20] They are widely studied to understand the cancer pathway.

In the present study, the tissue samples were chosen as the miRs have a specific tissue pattern of expression and it is considered reliable compared to others. Saito *et al*.[21] stated that OL has higher tendency to undergo malignant

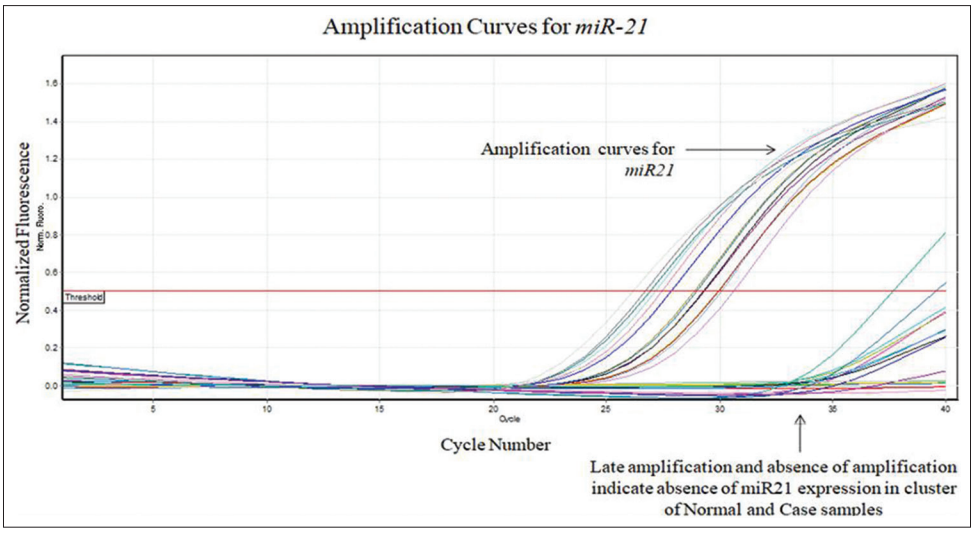

Figure 3: Amplification curve for miR-21

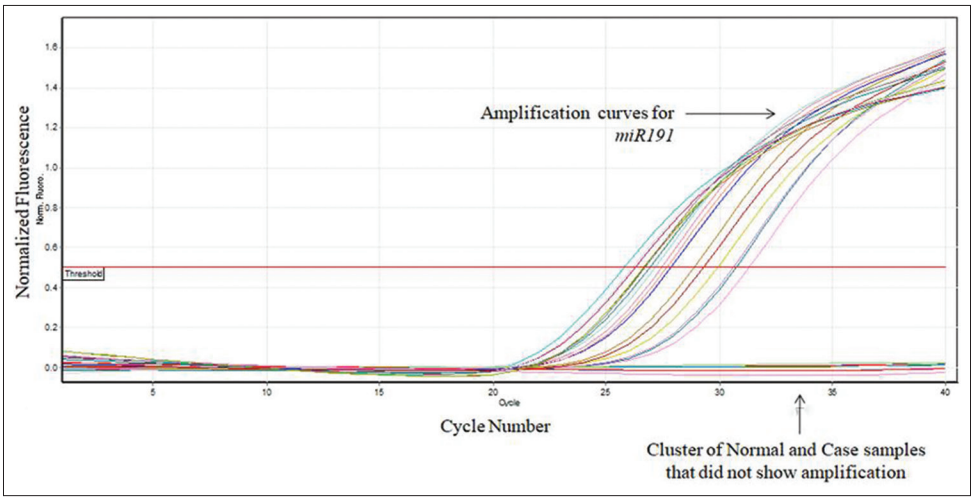

Figure 4: Amplification curve for miR-191

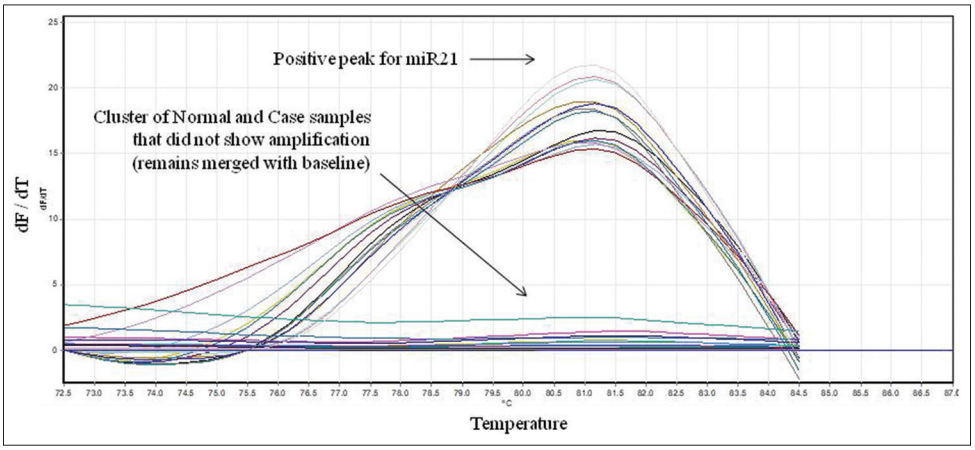

Figure 5: Melt Curve Analysis for miR-21

transformation. The literature search revealed a paucity of research in evaluating the expression profile of miR-21 and miR-191 in tissue samples of OL and oral squamous cell carcinoma in comparison with healthy individuals.

The study group involved patients aged from 40 to 70 years of age, as the incidence of OL is higher in between the 4th–7th decade of life and with male predominance.[22] The participants in the study were males in majority as tobacco

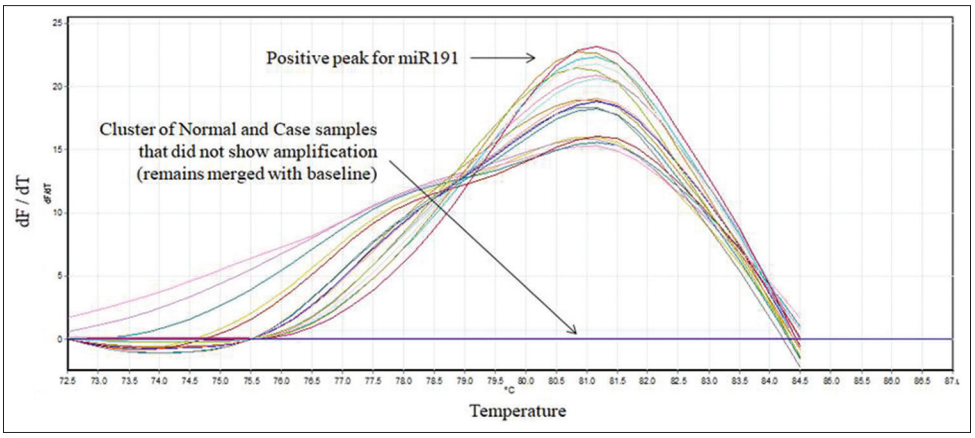

Figure 6: Melt curve Analysis for miR-191

Table 1: The intergroup comparison between three tissue types for the expression of microRNA-21 and microRNA-191 was assessed using Kruskal-Wallis test

Group	miR-21		miR-191	
	Obs	Rank sum	Obs	Rank sum
Normal	15	270.00	15	255.00
OL	15	307.00	15	372.00
OSCC	15	458.00	15	408.00

OL: Oral leukoplakia, OSCC: Oral squamous cell carcinoma, miR: MicroRNA

usage is more prevalent among males in India. Liu *et al.*[23] stated that the expression of miRs occurs in various forms of cancer, hence our study samples were recruited with no history of treated or untreated malignancies of any other site and system.

The traditional methods to detect miR are northern blotting, quantitative real-time PCR (qRT-PCR), next-generation sequencing, and microarray-based hybridization.[24] Considering the sensitivity, specificity, reliability, ease of use, precision, and accuracy, the quantitative real-time PCR was chosen to estimate the miR-21 and miR-191 in the collected tissue samples.[25]

In the present study, 15 patients with OL, 15 patients with oral squamous cell carcinoma, and 15 healthy individuals were chosen to study the expression of mi-21 and miR-191 among them. We also utilized age- and gender-matched tissues to minimize variations in gene expression caused by individuals.

According to Chang *et al.*,[9] Avissar *et al.*,[26] and Gombos *et al.*,[27] there is a notable positive expression of miR-21 in oral squamous cell carcinoma in comparison with healthy tissues.

Gombos *et al.*,[27] and Gissi *et al.*,[28] stated that there is a noteworthy presence of miR-191 in oral squamous cell carcinoma tissues when compared to the healthy tissue. Our results revealed that the expression of miR-21 and miR-191 was upregulated in oral squamous cell carcinoma compared to healthy tissues and it was statistically significant (P = 0.0005 and P = 0.0094, respectively). Hence, the present study result correlates with previous literature.

Cervigne *et al.*,[29] and Brito *et al.*,[30] revealed that there is a significant upregulation of miR-21 in OL in comparison with healthy tissues. We have observed that although there was an elevation in the expression of miR-21, there was no statistical significance (P = 0.7196). Such variations in expression could be attributed to the relatively smaller sample size as the current study was a pilot study.

Cervigne *et al.*,[29] Brito *et al.*,[30] and De Sarkar *et al.*[31] revealed that there is a significant overexpression of miR-21 in oral squamous cell carcinoma over healthy tissues. The present study results revealed a significant (P = 0.0059) overexpression of miR-21 in oral squamous cell carcinoma in comparison with OL. Hence, the present study result correlates with previous literature.

There is no reported evidence of research, analyzing the expression of miR-191 in OL tissue and to compare them with oral squamous cell carcinoma and healthy tissue, which is the primary hypothesis of the present study. The study revealed that there was a significant upregulation in the expression of miR-191 in OL (P = 0.0460) in comparison with normal tissues and there was no statistically significant overexpression of miR-191 (P = 0.7804) in oral squamous cell carcinoma in comparison with OL.

Oral leukoplakia samples collected in the present study were hyper-ortho/para keratosis with mild dysplasia and moderate dysplasia. These were subjected to Pearson's correlation coefficient of histopathological grading for micro RNA-21 and micro RNA-191 which revealed -0.2764 and -0.2543 respectively indicating a weak negative correlation. Brito *et al.*,[30] stated that there is minimal correlation with the grades of dysplasia and micro RNAs and in the present study a significant relationship between grades of dysplasia and leukoplakia was not elucidated. The present study had

Table 2: The results of hypothesis testing for microRNA-21 and microRNA-191 levels in different tissues using Dunn's *post hoc* analysis

Type of tissue	miR-21		miR-191	
	Column mean - row mean	*P*	Column mean - row mean	*P*
OSCC versus healthy tissue	3.590960	0.0005	2.732579	0.0094
OL versus healthy tissue	0.706731	0.7196	2.179619	0.0460
OSCC versus OL	2.884228	0.0059	0.64296	0.7804

OL: Oral leukoplakia, OSCC: Oral squamous cell carcinoma, miR: MicroRNA

Table 3: The Pearson's correlation between histopathological staging of oral leukoplakia and the expression of microRNA profile microRNA-21 and microRNA-191

	hp	miR-21	miR-191
Hp	1.0000		
miR-21	-0.2764	1.0000	
miR-191	-0.2543	0.5587	1.0000

relatively smaller samples in each grade of leukoplakia hence larger samples are required for validation. Hence, in our study, we have not elicited the changes based on the grades of dysplasia.

Chen *et al.*[32] compared the variation in the expression of miRs in between all the tissues, and it was significant. Hence, in our present study, the Kruskal–Wallis intergroup comparison revealed a significant variation in the expression of miR-21 ($P = 0.0007$) and miR-191 ($P = 0.0169$), respectively, which shows that these miRs play a significant role in the pathogenesis of oral squamous cell carcinoma.

The present study has various strengths along with limitations. The positive aspects of our miR profiling were carried out in tissue samples which are very specific over serum and saliva, and it was carried out in qRT-PCR which was the most reliable technique for miRNA evaluation. The study stands novel as there is no reported literature evaluating the miR-191 in OL tissue samples and showed a significantly upregulated expression of miR-191 in OL and oral squamous cell carcinoma when compared to normal tissues. Hence, this could be added to the "oncogenic miR profile."

The limitation of the present study was that we used a very small sample as it was a pilot study. Further research with larger samples at multicentric level is required to validate and generalize our results. Evaluation of miR along with the expression of their target gene could provide a better perception of their role in the pathogenesis of oral squamous cell carcinoma.

Conclusion

The altered expression of miR-21 was observed in oral squamous cell carcinoma. The altered expression of miR-191 was observed in oral squamous cell carcinoma and OL. Thus, we conclude that the dysregulated expression of miRs can be used as a tumor marker that could determine the susceptibility of normal and premalignant tissues to transform into oral cancer. The expression pattern of miRs differed within the same tissue groups, which was due to the local factors such as duration and frequency of the habit. To standardize our results, further prospective study with a larger sample size in each group and with a target gene for the miRs should be carried out at multicentric levels.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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