Expression of miR-21 in Saliva of Oral Submucous Fibrosis

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Abstract: miRNAs are short (19-24 nucleotides in length ) non coding RNAs that regulate messenger RNA (mRNA)or proteins levels either by promoting mRNA degradation or by attenuating protein translation. miRNA are good biomarkers. Objectives: The present study was aimed to quantitatively investigate miRNA-21expression in saliva and exfoliative cells of osmf patients and to compare miRNA-21expression in saliva and exfoliative cells of osmf. Methods: A total no of 40 subjects were selected for the study, out of which 20 were controls and 20 were patients with oral submucous fibrosis. Based on the clinical and histopathological evaluation, these patients were confirmed for oral submucous fibrosis. The samples were subjected for analysis of the presence of miRNA using real-time PCR. Results: The results of the study showed that out of 20 study salivary and exfoliative cells samples, 14 salivary samples and 11 exfoliative cells showed the miRNA expression and whereas no control samples showed miRNA. After the analysis we found that the 70% of study sample showed positive results for miRNA in saliva whereas 55% in exfoliative cells.Clinical relevance: miRNAs in body fluids are currently extensively explored for their potential as non–invasive diagnostic tumour markers. Tumour specific miRNAs may improve oral and pre-cancer diagnosis & prognosis. saliva can be used as a potential sample for evaluating miRNA-21.

1. Introduction

Oral submucous fibrosis (OSF), first described in the early 1950s, is a potentially malignant disease predominantly seen in people of Asian descent. It is a chronic progressive disorder and its clinical presentation depends on the stage of the disease at detection.2 The etiology of the disease remains elusive till today. A variety of etiological factors have been suggested which include betel nut, capsiaicin, malnutrition and vitamin imbalance, autoimmunity, viral and genetic predisposition. Micro RNAs (miRNAs) are small non coding RNAs that mediate gene expression at the post transcriptional level by degrading or repressing target messenger mRNAs (mRNA). Each miRNA has the ability to regulate many target genes in humans modulating the levels of mRNAs. They are encoded by genes located in non coding regions or in introns of protein coding genes and have been associated with the pathogenesis of oral cancer.3 Over expression of oncogenic miRNAs result in reduced tumour suppressor proteins and loss of tumour suppressor miRNA expression may cause elevated levels of oncogenic protein which will ultimately result in impaired apoptosis, cell differentiation, increased proliferation and metastasis. (Gomes and Gomez, 2008).26 Much of the work carried out using miRNA in head and neck cancer has used cancer cell lines and micro array technology (Tran et al, 2007; Hebert et al, 2007; Kozaki et al, 2008).26,27 miR-21 was first noted as an apoptotic suppressor in various cell lines. In a subsequent large scale study from 540 human samples, it was found that miR-21 is the only miRNA that is over expressed in six solid cancers including that of lung, breast, stomach, prostate, colon and pancreas.44 New evidence suggests that miRNA from cell lines may not show the same profile of the original tumour due to invitro growth conditions affecting miRNA expression. miRNA profiles also appear to be affected by tumour site which has not been controlled for in the majority of studies where cell lines comprising tonsillar tumours, hypopharynx and oral cavity and have been combined.23

2. Literature Survey

Saliva is used as a diagnostics medium for oral squamous cell carcinoma and saliva analytes such as proteins and DNA have been used to detect OSCC According to the li y et al 2004 thousands of miRNA are present in saliva and a panel of saliva miRNA can be used as oral cancer detection. The salivary mRNA seem to enter the oral cavity through various sources, including the 3 major salivary glands, gingival crevice fluid and desquamated oral epithelial cells. The majority of saliva mRNA seem to be present as partially degraded forms. These degraded forms mRNA maintain their stability in saliva through their association with unidentified macromolecules. Lu j et al stated that the miRNA serve important functions in cell growth ,differentiation, apoptosis, stress response, immune response and glucose secretion. Stadler et al stated that miRNAs are differentially expressed in various cancer compared with normal cells and it seems that miRNAs more accurately cluster different types of solid tumours than mRNA, suggesting that miRNAs can be used to detect the cancer. Fold change in miRNA between cancer cells and normal cells is relatively small, whereas the expression level of many miRNAs exhibits fold changes of ten to hundreds.20,30 Park et al detected the presence of miRNAs in both the whole and supernatant saliva, and demonstrated that two miRNAs, miR-125a and miR-200a, were lowered in the saliva of oral squamous cell carcinoma patients than in control subjects26.Recent studies have also identified both mRNA and miRNA in 2 types of cell derived lipid vesicles; microvesicles and exosomes. Microvesicles are relatively large vesicles released from the cell through blabbing. Exosomes are similar vesicles released when endosomally derived multivesicular bodies fuse with the plasma membrane.27 miRNA have been identified in both exosomes and microvesicles derived from a sources, including human and mouse mast cells, glioblastoma tumours, plasma, saliva and urine20

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Li et al first demonstrated that miRNA profile reflects the developmental lineage and differentiation state of the tumors through a global analysis of miRNA expression levels in hundreds of clinical samples. Compared with normal tissues, cancerous tissues generally tend to express smaller amounts of miRNAs, suggesting that miRNA signature is a promising tool to classify human cancers.27

Calin et al stated that miRNA expression profiling have been shown the miRNA expression levels are altered in primary human tumours. Lu et al stated that significant signatures of miRNA expression profiles can be linked to various types of tumours suggesting that miRNA profiling has diagnostic and perhaps prognostic potential. Certain miRNA can be tumour suppressor because of loss of these miRNA is often associated with cancers.27

Only one study to date (Cervigne et al, 2009) has looked at the role of miRNA in malignant transformation of oral dysplasia. A micro RNA signature associated with progression of oral leukoplakia to oral carcinoma was identified using fixed formalin paraffin embedded tissue using samples from 12 patients with progressive dysplastic leukoplakia and 4 with non progressive dysplastic leukoplakia.2

3. Materials and Methods

Selection of Patients
The study protocol was approved by Ethics committee for student proposal, Sri Ramachandra university and with the Helsinki Declaration of 1975 that was revised in 2000. Sample size was calculated and total no of 40 subjects were selected for the study, out of which 20 were controls (Group A) and 20 were patients with oral submucous fibrosis (Group B). All 40 patients were those of who reported to the department of oral medicine and radiology. Based on the clinical and histopathological evaluation, these patients were confirmed for oral submucous fibrosis. The patient age group for the oral submucous fibrosis, ranged from 21 to 67 years with mean age of 44. Out of 20 osmf patients one of them was female, 19 of them were males. All patients included in the study group were those who had not undergone any form of therapy for the presenting illness. Samples from 20 controls who has participated in the study were taken from the healthy volunteers of matched age and gender, who are free of oral and medical illness. without any habits of smoking, alcohol, chewing betel nut and tobacco. The age group for control ranged from 21 to 60 years with mean age of 40 years. The participants were instructed to rinse the mouth with tap water for 10 seconds before collection. The affected buccal mucosa was scraped by simple counter pressure by twirling the brush while moving it downward and counter pressure was applied with fingers against the cheek for 30 seconds and the brush was stored in a 2ml eppendroff tube directly without additional processing in -80 degree centrifuge freezer. Same method was followed for the control group.

Real Time PCR Amplification
cDNA, nuclease free water and SYBR green master mix are placed on ice and thawed for 15-20 min. SYBR green master mix was mixed by pipetting up and down. The rest of the reagents are mixed by vortexing and Spun down. Immediately after use, cDNA template are diluted for the planned real-time PCR reactions 80x in nuclease free water. 395μl nuclease free water to each 5 μl of reaction. Multiple real time PCR reaction were performed with the same microRNA primer set. Later the required amount of PCR master mix was prepared and placed on ice. It was recommended to include all excess reagents in the master mix to compensate for pipetting excess material. Then, placed the relevant volume of PCR master mix in PCR tubes. The reaction was mixed by gentle pipetting to ensure that all reagents were thoroughly mixed. Later it was incubated for 60 min at 42 c and heat was inactivated in the reverse transcriptase for 5 min at 95 c. Immediately cooled to 4 c and stored at 4 c.

First Strand CDNA Synthesis
Each of the templates RNA samples were adjusted to concentration of 5ng/μl using nuclease free water. Reagents were prepared and gently thawed at the 5x reaction buffer and nuclease free water and immediately placed on ice mixed by vortexing. Later RNA spike was resuspended in by adding 40 μl nuclease free water to the tube and again mixed by vortexing and left on ice for 15-20 minutes. Then enzyme was mixed by flicking the tubes and placed on ice. The reagents were spun again. Multiple RNA samples were prepared in RT mastermix of the 5x reaction. Buffer, water, enzyme were mixed and prepared. The required amount of RT master mix was prepared and Placed on ice. The reaction were mixed by very gentle vortexing or pipetting to ensure that all reagents were thoroughly mixed. Later it was incubated for 60 min at 42 c and heat was inactivated in the reverse transcriptase for 5 min at 95 c. Immediately cooled to 4 c and stored at 4 c.

Real Time PCR
A real-time PCR reaction mixture of 20 μl prepared (including template) was added in 96 well plate and gently mixed. Non template control (NTC without template only SYBR and primers) added in different well to check the purity of the same.

Sampling of Saliva
2 ml of saliva were collected by spitting method in collecting tube, in a 2 ml sterile eppendorff tube from 20 control group and 20 OSMF patients. The salivary samples collected were transferred to the laboratory and was stored at-80 degree centigrade.

Sampling of Exfoliative Cytology
Buccal cell sample collection was done with the help of cytobrush from 20 control group and 20 OSMF patients. All
All the samples are loaded and linked to the plate of the equipment (7900 HT Fast Real Time PCR) with the following PCR conditions. The results were analysed with the RQ manager (CT calculated).

4. Results

The expression of miR-21 was studied in both saliva and exfoliative cells in normal & also in biopsy proven oral submucous fibrosis. The study group A (control) comprised of 20 cases with age group 21 to 60 years with mean age of 40. The study group B (OSMF) comprised of 20 cases with age group 21 to 67 years with mean age of 44. These group showed 19 males and 1 female.

Table 1: microRNA expression in saliva of study group A

<table>
<thead>
<tr>
<th>S.No</th>
<th>Contents</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SYBR Green mix (2x)</td>
<td>10 µl</td>
</tr>
<tr>
<td>2</td>
<td>Forward primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>3</td>
<td>Reverse primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>4</td>
<td>Template</td>
<td>1 µl</td>
</tr>
<tr>
<td>5</td>
<td>Sterile water</td>
<td>7 µl</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Table 1 shows miRNA expression in saliva of study group A. miRNAs were undetermined in all 20 salivary samples.

Table 2: microRNA expression in saliva of study group B

Out of 20 samples in group B, 14 samples showed miRNA expression and no values were obtained from 6 samples. The highest expression of miRNA was obtained 38.22674 and the lowest expression of miRNA was 28.70719. The mean value is 34.597554.

Table 2 shows miRNA expression in saliva of study group B

<table>
<thead>
<tr>
<th>Sample</th>
<th>miRNA expression</th>
<th>Sample</th>
<th>miRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1</td>
<td>Undetermined</td>
<td>PS2</td>
<td>32.3682</td>
</tr>
<tr>
<td>PS3</td>
<td>32.3682</td>
<td>PS4</td>
<td>36.66063</td>
</tr>
<tr>
<td>PS5</td>
<td>38.19478</td>
<td>PS6</td>
<td>35.52382</td>
</tr>
<tr>
<td>PS7</td>
<td>Undetermined</td>
<td>PS8</td>
<td>35.86247</td>
</tr>
<tr>
<td>PS9</td>
<td>35.20609</td>
<td>PS10</td>
<td>32.61348</td>
</tr>
<tr>
<td>PS11</td>
<td>31.96936</td>
<td>PS12</td>
<td>Undetermined</td>
</tr>
<tr>
<td>PS13</td>
<td>Undetermined</td>
<td>PS14</td>
<td>38.22674</td>
</tr>
<tr>
<td>PS15</td>
<td>33.45791</td>
<td>PS16</td>
<td>34.97822</td>
</tr>
<tr>
<td>PS17</td>
<td>28.70719</td>
<td>PS18</td>
<td>37.43601</td>
</tr>
<tr>
<td>PS19</td>
<td>33.16087</td>
<td>PS20</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: microRNA expression in exfoliative cells of study group A

Table 3 shows miRNA expression in exfoliative cells of study group A. miRNAs were undetermined in 19 samples and 1 sample value of 39.89007.

Figure 1 indicates that the highest expression of miRNA levels obtained was 38.22674 and the lowest expression of miRNA was 28.70719.

Table 3 shows miRNA expression in exfoliative cells of study group A.
Table 4: microRNA Expressions in exfoliative cells of study group B

<table>
<thead>
<tr>
<th>PE1</th>
<th>PE2</th>
<th>PE3</th>
<th>PE4</th>
<th>PE5</th>
<th>PE6</th>
<th>PE7</th>
<th>PE8</th>
<th>PE9</th>
<th>PE10</th>
<th>PE11</th>
<th>PE12</th>
<th>PE13</th>
<th>PE14</th>
<th>PE15</th>
<th>PE16</th>
<th>PE17</th>
<th>PE18</th>
<th>PE19</th>
<th>PE20</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.13953</td>
<td>Undetermined</td>
<td>33.98576</td>
<td>32.72281</td>
<td>31.18897</td>
<td>Undetermined</td>
<td>38.41941</td>
<td>Undetermined</td>
<td>Undetermined</td>
<td>36.63158</td>
<td>Undetermined</td>
<td>33.85208</td>
<td>34.64386</td>
<td>36.87119</td>
<td>Undetermined</td>
<td>30.54784</td>
<td>Undetermined</td>
<td>Undetermined</td>
<td>Undetermined</td>
<td>32.88863</td>
</tr>
</tbody>
</table>

Table 4 shows miRNA expression in exfoliative cells of study group B

Out of 20 samples, 9 samples showed undetermined value and 11 samples showed the miRNA levels. The highest expression of miRNA obtained was 38.41941 and the lowest expression of miRNA was 30.54784. The mean value was 34.171970.

Figure 3: miRNA expression in exfoliative cells of study group B

Figure 3: Indicates that the highest expression of miRNA obtained was 38.41941 and the lowest expression of miRNA was 30.54784.

Figure 5: Comparison of mean value between saliva & exfoliative cells of study group B

Figure 5: Indicates a marginal increase in the mean value of saliva when compared with exfoliative cells. The mean value of saliva & exfoliative cells is 34.597554 & 34.171970.

5. Discussion

The current study was done to evaluate the expression of miR 21 in saliva and comparing it with exfoliative cells of 20 oral submucous fibrosis patients and with 20 healthy individuals. The present study was aimed for the detection of miRNA in saliva & exfoliative cells of OSMF patients.

miRNAs are found significant number outside of the cells, including various body fluids. These are stable & show distinct expression profiles among different fluid types. miRNA profile reflects the developmental lineage and differentiation state of the tumors through a global analysis of miRNA expression levels in hundreds of clinical samples. Compared with normal tissues, cancerous tissues generally tend to express smaller amounts of miRNAs, suggesting that miRNA signature is a promising tool to classify human cancers.9

According to nilva k cervigne, calin and li ye and Danielson et al miRNAs demonstrated significant over expression in leuokplakia & oral squamous cell carcinoma compared with normal. In case of OLP patients, miR -21 showed significantly increased levels when compared to normal oral mucosa. Thousands of miRNA are present in saliva & these miRNAs can be used as oral cancer detection.2 The salivary miRNA seems to enter the oral cavity through various sources are 3 major salivary glands, gingival crevicular fluid & desquamated oral epithelial cells.

The majority of saliva mRNA seems to be present as partially degraded forms. These degraded forms mRNA maintain their stability in saliva through their association with unidentified macromolecules. St john MA, Li y zou et al 2004 stated that salivary mRNAs can be used as biomarkers for oral cancer that combined measurement of 7 different mRNAs showed a specificity & sensitivity of 0.91 for oral cancer.21,19

Li et al. found elevated levels of miR-21 in OSCCs of the tongue, thus relating this miRNA and cell growth, apoptosis, and tumorigenesis. They found that miR-21 over expression was inversely correlated with levels of tropomyosin1 and phosphatase tensin homologue, two apoptotic markers. A multivariate analysis revealed that miRNA-21 is an independent predictor of OSCC.27

According Fu et al, showing that miR-21 over expression is associated with a worse average survival rate of carcinomas, in contrast to those that do not show over expression, and a shorter disease-free period, thus confirming the prognostic value of this miRNA.22
Stadler et al. stated that miRNAs are differentially expressed in various cancer compared with normal cells. Fold change in miRNA between cancer cells and normal cells is relatively small, whereas the expression of many miRNAs exhibits fold changes of ten to hundreds, suggesting that miRNAs can be used to detect the cancer.\(^1\)

The present study was carried out using salivary sample which yielded the similar result to that of Park et al. who detected over 50 stable miRNAs in whole saliva and supernatant in OSCC patients, while miR-125a and miR-200a were significantly subexpressed, compared to healthy control patients. It seems that the degree of degradation of miRNAs in endogenous saliva is slower than that of exogenous miRNAs. It appears that methylation analysis for miRNAs in saliva is also an effective tool for diagnostics and prognostics.\(^2\)

In this study miRNA analysis was done using Real time PCR, it has been proved that it is a superior method for miRNA expression analysis due to sensitivity and specificity of the PCR.

In present study, comparison of OSMF patients against controls have shown that salivary sample is showing high positive results that is 70% of miRNA expressions whereas analysis of exfoliative cells revealed significant positive value that is 55%. So, Out of 20 osmf salivary samples, 14 samples showed miRNA expressions & 6 samples showed undetermined value and 20 osmf exfoliative cells sample, 14 samples showed miRNA levels and 9 samples showed undetermined value and out of 20 control salivary samples, all the samples showed undetermined values and in 20 exfoliative cells sample, 19 samples showed undetermined value and 1 sample showed expression for which the reasons may be due to technical /sampling error.

6. Summary and Conclusion

The present study was conducted to evaluate the expression of miRNA -21 in saliva and comparing it with exfoliative cells of oral submucous fibrosis. The analysis of miRNA was done using Real time PCR method. The study group comprised of 20 patients with mean age of 44 yrs and all the patients who were clinically diagnosed as oral submucous fibrosis were confirmed histopathologically and 20 controls, age and sex matched with mean age of 40 yrs were included in the study for collection of salivary and exfoliative cells samples.

The results of the study showed that out of 20 study salivary and exfoliative cells samples, 14 salivary samples and 11 exfoliative cells showed the miRNA expression and whereas no control samples showed miRNA. After the analysis we found that the 70% of study sample showed positive results for miRNA in saliva whereas 55% in exfoliative cells.

Though high cost, regulated availability of high end equipment and limited sample size may be a limitation to draw a conclusion, however the present study confirmed the abundant expression of miRNA in saliva and hence saliva can be used as a potential sample for evaluating miRNA-21.

miRNAs in body fluids are currently extensively explored for their potential as non-invasive diagnostic tumour markers. Tumour specific miRNAs may improve oral and pre-cancer diagnosis & prognosis, since several promising miRNAs have already been described as non invasive biomarkers for different tumour entities.

Compliance with Ethical Standards

Conflict of Interest: Author A declares that he has no conflict of interest. Author B declares that he has no conflict of interest. Author C declares that he has no conflict of interest.

Funding: The work was supported by the Department of Oral Medicine and Radiology, Sri Ramachandra Dental College, Sri Ramachandra University Chennai, India

Ethical Approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional.

Informed Consent: For this type of study, Informed consent was obtained from all individual participants included in the study.

References


[43] Chun-ju yang, Wilma Grace Shen. miR-221 and miR-222 expression increased the growth and tumorigensis of oral carcinoma cells. *J Oral Pathol 2011 40,: 560-566.*


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