Clinicopathological correlation of microRNA 21 expression in oral cancer patients with tobacco chewing habits

Pragya Khanna and Nishi Tandon

Keywords: miRNA21, oral cancer, tobacco chewing, oral leukoplakia, polymerase chain reaction

Introduction

Oral cancer is one of the most common cancers globally. Malignancies of oral cavity and oropharynx are responsible for 3% of total cancers in men and 2% of total cancers in women [1]. India, along with some other South Asian countries is a high risk region for oral cancer due to highly prevalent habit of tobacco chewing [2]. Tobacco chewing, pan masala and Gutkha use have been shown to be etiological factors behind a number of malignant, premalignant and non-malignant lesions of oral cavity [3-5]. MicroRNAs (miRNA), a class of small (18-24 nucleotides), non-coding regulatory RNAs play an important role in induces autophagy, apoptosis, and activation of the PTEN/ERK axis, particularly among tobacco users/smokers [6]. In effect they may function as tumor suppressors and oncogenes [7]. They are also differentially expressed in various types of cancers including oral cancer, compared with noncancerous tissues, suggesting that they may have crucial roles in tumorigenesis [8]. There are various tumor suppressor and oncogenic miRNAs. miR-15a, miR16-1 that have been identified as tumor suppressor miRNAs and miR-155, miR-17-92 cluster, miR-21, miR-372, miR-373 that have been identified as oncogenic miRNAs [9, 10]. Considering the possible relationship between miRNA-21 expression and progression of oral cancer, the present study was planned to correlate microRNA-21 expression with clinic pathological profile of tobacco chewing related oral cancer patients.

Material and Method

The present study was carried out as a case-control study, for a two year period in which a total of 20 biopsy tissue specimen obtained from oral cancer patients having tobacco chewing habits were enrolled as cases and an equal number of biopsy tissue specimen obtained from age, and sex-matched patients of oral leukoplakia were enrolled as controls. Clinical details, personal habits were noted and histopathological assessment was done. miRNA21 was done using Taq Man microRNA assay (ABI) using Polymerase Chain Reaction (PCR). Real time PCR results was analyzed and expressed as relative miRNA expression of the threshold cycle (CT) values.

Results

Mean age of cases was 52.65±7.82 years, majority of cases were males (85%). Among cases, majority (70%) had clinical stage I and were histo-pathologically well differentiated (75%). There were 3 (15%) cases with metastasis. Mean relative miRNA 21 expression were significantly higher in cases (10.53±3.52) as compared to that in controls (8.76±1.16) (p=0.029). Relative miRNA 21 expression was able to differentiate between leukoplakia and oral cancer cases. miRNA21 also showed a significant correlation with clinicopathological factors.

Conclusion

miRNA21 expression was able to differentiate between leukoplakia and oral cancer cases. miRNA21 also showed a significant correlation with clinicopathological factors.

Abstract

MicroRNA 21 (miRNA-21) has been identified as oncogenic RNA, implicated in etio-pathogenesis of various neoplastic lesions. Considering the possible relationship between miRNA-21 expression and progression of oral cancer, the present study was planned to correlate microRNA-21 expression with clinic pathological profile of tobacco chewing related oral cancer patients.
Material and Methods
Biopsy specimen obtained from oral mucosa of 20 patients of clinically and histopathologically confirmed oral cancer having tobacco use history and a total of 20 age and sex-matched patients of oral leukoplakia were enrolled as cases and controls, respectively, from May 2013 to May 2015 at this institution. Prior approval was obtained from the Institutional Ethics Committee and informed consent was taken from all the participants.

Inclusion criteria: Patients with clinically and histopathologically confirmed, cases of squamous cell carcinoma of oral cavity and having history of oral tobacco use, in any form.

Age and sex matched, clinically and histopathologically confirmed patients of oral leukoplakia as controls.

Exclusion Criteria: Patients or controls who had undergone therapy including chemo-radiation, for the above conditions. Detailed clinical history especially with reference to use of oral tobacco products in any form and duration of tobacco use was obtained from case record for both the groups. Formalin fixed paraffin embedded sections of biopsy specimens were studied using hematoxylin & Eosin stain for assessment of histopathological grade. Metastasis if any was noted.

Real Time RT-PCR Quantification of miRNA-21
Fresh frozen tissue was from same patients and controls was collected in RNA later (Thermo-Fischer), followed by RNA extraction by Taq Man microRNA assays (ABI), to detect expression levels of mature microRNA-21. For reverse transcription (RT) reactions, 10ng total RNA was used in each reaction (5 microlitres) and mixed with RT primer (3 microlitres). RT reactions were carried out at 16 °C for 30min, 42 °C for 30 min and 85 °C for 5min, then maintained at 4 °C. Following RT reactions, 1.5 microlitre cDNA was taken up for polymerase chain reaction (PCR) along with Taq Man primers (2 microlitres). PCR was conducted at 95 °C for 10min followed by 40 cycles at 95 °C for 15sec and at 60 °C for 60 sec in the ABI Step one plus real-time PCR system. Real time PCR results was analyzed and expressed as relative miRNA expression of the threshold cycle (CT) values. U6B was used for normalization.

Relative miRNA expression was calculated as follows
\[ R = 2^{\Delta \Delta C_t} \]

Where, \( \Delta C_t \) is the difference in \( C_t \) values of reference and sample/control.

Statistical Evaluation
The data was subjected to statistical analysis using Statistical Package for Social Sciences, Version 15.0. Chi-square test was used for comparing proportions. Mean values were compared using Independent samples ‘t’-test and ANOVA. Confidence level of study was kept at 95%. ‘p’-value less than 0.05 indicated a statistically significant association.
significantly overexpressed in gastric cancer patients. More importantly, they also showed that forced expression of miRNA-21 significantly enhanced cell proliferation and invasion in AGS cells, a human gastric cancer cell line, whereas knockdown of miRNA-21 by inhibitor caused a significant reduction in cell proliferation and a significant increase in apoptosis. Furthermore, they demonstrated that knockdown of miRNA-21 significantly decreased cell invasion and migration of AGS cells. miRNA 21 has also been identified as a useful diagnostic, prognostic and therapeutic biomarker in oral cancer patients.

In line with the above quoted studies, in our study we found that miRNA21 expression to be significantly correlated with higher clinical stages, higher histopathological grades and metastasis, thus showing that miRNA21 expression was related with progression of oral cancer in these patients. Similar to the findings of the present study, Tseng et al. also found a significant association of high expression of miRNA21 to be associated with oral cancer metastasis. In their study, higher expression of miRNA was also seen in higher clinical and pathological grades, but they did not find this relationship to be significant statistically. The reason for this discrepancy might be use of categorical rather than quantified evaluation in the present study. The findings in present study documented a significant causal pathogenetic relationship between oral cancer and miRNA-21. Despite being a pilot study, it provided useful information regarding progression and transformation of tobacco related oral precancerous conditions to malignant conditions and their increasing severity. Further longitudinal studies on a larger sample size are suggested to explore the potential of miRNA-21 expression in prognosis and prediction of disease-free survival of oral cancer patients at advanced stage.

### Table 1: Comparison of Demographic, Clinical Profile and miRNA21 expression between Cases and Controls

<table>
<thead>
<tr>
<th>SN</th>
<th>Variable/Parameter</th>
<th>Cases (n=20)</th>
<th>Controls (n=20)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mean age±SD (Range) years</td>
<td>52.65±7.82 (34-65)</td>
<td>46.75±11.20 (28-66)</td>
<td>t=1.932; p=0.061</td>
</tr>
<tr>
<td>2.</td>
<td>Male: Female</td>
<td>17 (85%); 3 (15%)</td>
<td>13 (65.0%); 7 (35.0%)</td>
<td>χ²=2.133; p=0.144</td>
</tr>
<tr>
<td>3.</td>
<td>Smokers/tobacco users</td>
<td>13 (65.0%)</td>
<td>2 (10.0%)</td>
<td>χ²=12.91; p&lt;0.001</td>
</tr>
<tr>
<td>4.</td>
<td>Tobacco chewers</td>
<td>7 (35.0%)</td>
<td>4 (20.0%)</td>
<td>χ²=8.29; p=0.004</td>
</tr>
<tr>
<td>5.</td>
<td>Tobacco use &gt;10 years</td>
<td>15 (75.0%)</td>
<td>3 (15.0%)</td>
<td>χ²=14.54; p&lt;0.001</td>
</tr>
<tr>
<td>6.</td>
<td>Tobacco use &gt;10 sticks/pints per day</td>
<td>8 (40.0%)</td>
<td>1 (5.0%)</td>
<td>χ²=7.03; p=0.008</td>
</tr>
<tr>
<td>7.</td>
<td>Mean miRNA21±SD</td>
<td>10.53±3.52</td>
<td>8.76±1.16</td>
<td>t=2.263; p=0.023</td>
</tr>
</tbody>
</table>

### Table 2: Clinicopathological profile of Oral Cancer cases and their relationship with miRNA21 expression (ΔG - Relative Fold-Rise)

<table>
<thead>
<tr>
<th>SN</th>
<th>Variables</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Clinical Stage</td>
<td></td>
<td></td>
<td></td>
<td>F=17.04; p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>14</td>
<td>5.95</td>
<td>11.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>3</td>
<td>34.66</td>
<td>34.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>2</td>
<td>67.75</td>
<td>44.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1</td>
<td>128.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
<td>t=9.336; p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>17</td>
<td>8.85</td>
<td>13.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3</td>
<td>100.22</td>
<td>27.61</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Histopathological grade</td>
<td></td>
<td></td>
<td></td>
<td>F=12.68; p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Well differentiated</td>
<td>15</td>
<td>9.92</td>
<td>20.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mod. differentiated</td>
<td>4</td>
<td>43.49</td>
<td>38.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>1</td>
<td>128.27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Conclusion

In the present study, we found a significant correlation of miRNA expression in pathogenesis and progression of oral cancer particularly in patients having history of oral tobacco use. However, further studies with larger sample size are needed to confirm the definitive role in pathogenesis as well as prognostic value of miRNA in oral neoplasms in both tobacco as well as non-tobacco using oral cancer patients.

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### Conflicts of Interest: There are no conflicts of interest.

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### References


