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

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Abstract
Introduction: Oral cancer is one of the most common malignancies and has a significant association with tobacco chewing. Micro RNA 21 (mi-RNA 21) has been identified as oncogenic RNA, implicated in etio-pathogenesis of various neoplastic lesions.
Objective: (i) To assess the microRNA 21 expression in oral cancer patients with history of tobacco chewing habits and (ii) to carry out its clinicopathological correlation.
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. Mean values were compared using independent samples 't'-test and ANOVA using SPSS 21.0 software. p-value less than 0.05 indicated a statistically significant association.
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 miRNA21 values showed a significant increasing trend with increasing clinical stage, histopathological grade and metastasis.
Conclusion: miRNA21 expression was able to differentiate between leukoplakia and oral cancer cases. miRNA21 also showed a significant correlation with clinicopathological factors.

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

Introduction
Oral cancer is one of the most common cancer forms 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 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

$R = 2^{-[\Delta C_t \text{ sample} - \Delta C_t \text{ control}]}$

Or

$R = 2^{-\Delta\Delta C_t}$

Where, ΔC_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.

Results

Age of cases ranged from 24 to 65 years. Mean age of cases was 52.65±7.82 years. Age of controls ranged from 28 to 66 years. Mean age of controls was 46.75±11.20 years. Majority of cases (85%) as well as controls (65%) were males. There was no statistically significant difference between cases and controls for age and sex (p>0.05). Significantly higher proportion of cases as compared to controls were smoker/tobacco users (65% vs 10%; p<0.001), tobacco chewers (35% vs 10%; p=0.004), tobacco users >10 years (75% vs 15%) and >10 sticks/pints per day (40% vs 5%) (p<0.05). Mean miRNA expression in terms of relative-fold rise (ΔC_t) values were significantly higher in cases (10.53±3.52) as compared to that of controls (8.76±1.16) (p=0.023) (Table 1).

Majority of cases had clinical stage I (n=14; 70%) and did not have metastasis (n=17; 85%). Histopathologically, there were 15 (75%) well differentiated, 4 (20%) moderately differentiated and 1 (5%) poorly differentiated lesions. A significant incremental trend of ΔC_t values was observed with increasing clinical stage (<0.001)/. Mean ΔC_t values were significantly higher in cases with metastasis (100.22±27.61) as compared to those without metastasis (8.85±13.40) (p<0.001). Mean ΔC_t values were 9.92±20.66, 43.49±38.24 and 128.27 respectively in well differentiated, moderately differentiated and poorly differentiated cases (p<0.001).

Discussion

The present study used a relative fold rise of ΔC_t values in miRNA-21 expression of test samples against U6B as the reference. Mean ΔC_t values (Against U6B reference) of cases and controls were 10.53±3.52 and 8.76±1.16 respectively. Statistically this difference was significant indicating that the miRNA-21 expression is supposed to be higher in cases as compared to controls. On evaluating further relative fold rise based on mean ΔC_t values of cases and controls was 3.41 for cases as compared to controls. However, on taking mean value of control to be the criteria for relative fold rise for individual case samples, the mean fold rise in miRNA-21 expression was observed to be 22.25 in cases as compared to controls. Similar to findings of present study, Hu *et al.* in their study on patients with laryngeal cancer also found significantly higher relative fold rise of miRNA-21 in cases as compared to controls.[11] Altered expression of microRNA, including miRNA-21 in premalignant oral epithelial lesions such as leukoplakia, oral submucous fibrosis, oral lichen planus and some malignant carcinoma like oral squamous cell, verrucous, spindle cell, Merkel cell carcinoma and basal cell has been documented in context with their relevance in cancer development and progression [12]. A recent study that assessed salivary miRNA-21 levels in 36 healthy controls and compared it with 36 newly diagnosed oral premalignant lesions (Leukoplakia, oral submucous fibrosis, oral lichen planus and oral submucous fibrosis with leukoplakia) found that all the oral premalignant lesions had significantly higher expression of miRNA-21. Among different premalignant lesions, leukoplakia had maximum expression of miRNA-21, thereby showing a progressive nature of its expression with increasing potential of malignancy [13]. The findings of the present study are in consonance with these reports.

miRNA-21 has a definitive role in cancer pathogenesis and progression. In another study,miRNA-21 was found to be

significantly overexpressed in gastric cancer patients.[14] 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 [15, 16].

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 [17]. 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

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

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

SN	Variables	n	Mean	SD	Statistical significance
1.	**Clinical Stage**				
	I	14	5.95	11.71	F=17.04; p<0.001
	II	3	34.66	34.26	
	III	2	67.75	44.65	
	IV	1	128.27	.	
2.	**Metastasis**				
	No	17	8.85	13.40	t=9.336; p<0.001
	Yes	3	100.22	27.61	
3.	**Histopathological grade**				
	Well differentiated	15	9.92	20.66	F=12.568; p<0.001
	Mod. differentiated	4	43.49	38.24	
	Poorly differentiated	1	128.27	.	

Conclusion

In the present study, we found a significant correlation of mi-RNA 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 mi-RNA 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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