Original Article

Site specificity and expression profile of miR-21 in oral squamous cell carcinoma

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Abstract

Background: Oral Squamous Cell Carcinoma (OSCC) is the most common epithelial malignancy of the oral cavity which has evolved globally as a grave and growing health problem. It shares a wide geographic variation with respect to the incidence rate and exhibits anatomic adaptation to oral environment with varied clinical presentation along with a spectrum of histological mélange. Besides, in recent cancer research, both genetics and epigenetics add on at the molecular level and accounts for this diversification and tumor heterogeneity of OSCC and thereby substantiates to the miRNA expression profiling in OSCC.

Aims and Objectives: In the present study, subsite specificity of miR-21 expression in tissue specimens of OSCC of Tongue, Buccal mucosa, and Gingivo buccal (GB) sulcus were analyzed

Materials and Methods: Quantification of miR-21 was done on 30 tissue samples of OSCC using real-time polymerase chain reaction (RT-PCR).

Results: Results indicated that miR-21 expression was significantly expressed at the subsites. Out of 30 samples, 22 showed upregulation, and 8 showed down-regulation with reference to endogenous control. The comparative Ct method was used to analyze the differences in subsite specific expression of miR-21 in OSCC cases. It was significantly upregulated in the buccal mucosa ($p=0.002$), followed by GB sulcus ($p=0.01$) and Tongue ($p=0.25$).

Conclusion: In conclusion, the study could identify the differential miR-21 expression at sub-sites, indicating that it may serve as a diagnostic marker with further elaboration on a larger sample size..

Keywords: Biomarker, miR-21, oral cancer, oral squamous cell carcinoma, sub-site specificity

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INTRODUCTION

Oral cavity is a complex anatomical and functional compartment which harbors diverse soft-tissue components. Diversification can be attributed to embryological lineages, compositional variations and functional adaptations, which in turn explain the heterogeneity of oral squamous cell carcinoma (OSCC).

The soft-tissue sites of the oral cavity such as lips, gingiva, buccal mucosa, gingivobuccal (GB) sulcus, tongue and floor

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of the mouth are characterized by the unique set of mucosal and submucosal tissue composition. Hence, site-specific inter-tumoral heterogeneity is quite conceivable in OSCC.[1] The evolution and progress of cancer varies at sub-sites in the oral cavity and are influenced by growth pattern, clinical behavior, different risk factors and molecular (genetic and epigenetic) alterations.

The anatomic site of the tumor is an important factor for disease outcome and treatment. For instance, buccal mucosa represents the most common anatomic site for carcinoma development followed by carcinoma of the tongue in India.[2] Tongue squamous cell carcinoma (SCC) is well known for its high rate of proliferation and nodal metastasis.[3]

Micro ribonucleic acid (miRNA) is the gene specific regulator, involved in many essential biological activities such as cellular differentiation, proliferation and apoptosis and thus, their deregulation can affect normal cell growth and even participate in carcinogenesis.[4]

miRNAs possess unique properties such as abundant tissue expression, which allows for reproducible isolation and quantification. Distinct expression profiles of miRNA in OSCC offers the use of specific miRNA (s) signature for early stage diagnosis, prediction and prognosis.[5] The main advantage of using miRNA as a central diagnostic tool is that it is more stable and not degraded in formalin-fixed, paraffin-embedded (FFPE) tissue besides its specificity.[6]

miRNA expression profiles reflect the developmental origin of a tissue and are chiefly tissue and site specific in the head and neck. The probable reason could be the embryological events that result in the development and that is the reason for distinct anatomical sites containing dissimilar miRNA profiles.[6] Very few studies are focused upon this particular research field.

miR-21 is one of the most widely studied biomarker in head and neck SCC due to the experimental evidence on its inhibition of multiple tumor suppressor targets such as phosphatase and tensin homolog deleted on chromosome 10, Tropomyosin-1 and programmed cell death-4.[7] It has been shown to be overexpressed and regulate several biological functions in OSCC. The oncogenic role of miR-21, by promoting cell proliferation, invasion, antiapoptosis and chemoresistance is established by the number of *in vivo* and *in vitro* experiments.[4]

miRNA expression profiles differ between tumor and normal tissue in many types of cancer and its profiling are a promising field for finding new diagnostic and prognostic tools in SCC.

Literature search divulges that no independent studies of miRNA expression in OSCC of buccal mucosa, GB sulcus and tongue are conducted and also handful of studies are on comparative assessment miRNA expression among the sub-sites of the oral cavity. Hence, it is unclear if there exist any difference in miRNA expression in the sub-sites of the oral cavity.

The present study was conducted to analyze and quantify the sub-site specificity of miR-21 expression in the tissue specimens of OSCC of tongue, buccal mucosa and GB sulcus.

MATERIALS AND METHODS

Tissue sample characterization

A total of 30 OSCC tissue sample blocks were retrieved from Department of Oral Pathology of an Institution. After the Ethics Committee approval of the study protocol, OSCC tissue samples were categorized based on the site under the following groups.

- Group 1: Histologically confirmed cases of OSCC of the tongue ($n = 10$)
- Group 2: Histologically confirmed cases of OSCC of the buccal mucosa ($n = 10$)
- Group 3: Histologically confirmed cases of OSCC of GB sulcus ($n = 10$).

From the FFPE tissue blocks, 5 μ thick sections were cut. Ten sections from the blocks were used for RNA extraction, giving a total tissue area of approximately 1 cm^2[8] Tissue samples from the three study groups and healthy gingiva as the control group ($n = 10$) were collected in 2 ml microcentrifuge tube containing RNA later solution (GCC Biotech, West Bengal, India) and stored until RNA extraction.

Total RNA extraction

The RNeasy FFPE Kit (73504-Qiagen, Hilden, Germany) was used for the purification of total RNA from FFPE tissue sections following the manufacturer's manual. The concentration, purity and amounts of total RNA were quantified using Eppendorf Biophotometer plus spectrophotometry.

cDNA synthesis

Prime script Reverse transcription (RT) Reagent kit (RR037A-Takara, Japan) was used for cDNA synthesis and the procedure was followed according to manufacturer's protocol. cDNA reaction was prepared

according to the set protocol as follows: 2 µl of Prime script buffer, 0.5 µl RT enzyme, 0.5 µl of gene-specific reverse primer, 5 µl of total RNA and RNase water each. The reaction mixture was incubated at 42°C for 15 min and 85°C for 5 s followed by 4°C for 2 min.

cDNA was then stored at − 80°C until further analysis. The quantification of cDNA was recorded using Eppendorf bio photometer plus.

miR-21 expression

A set of oligonucleotide primers was used for the miR-21 gene expression study as described by Luo *et al*. [Table 1].[9]

Real-time polymerase chain reaction (RT-PCR) was performed using SYBR green quantitative PCR reagent kit (RR820-Takara, Japan) on 96 well thermal cycler. PCR volume for amplification included 12.5 µl SYBR premix 1 µl each of forward and reverse primer, 2 µl of cDNA solution and 8.5 µl of sterile water. The reaction mixture was run at 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 62°C for 30 s and 72°C for 30 s. Expression levels of miR-21 were analyzed with RT-PCR amplification and cycle threshold (Ct) values were recorded. U-6 was used as an endogenous reference control for data normalization.

Statistical analysis

Descriptive analysis of all the explanatory and outcome parameters was done using the mean and standard deviation for the quantitative variables and frequency and proportions for the categorical variables. One-way analysis of variance (ANOVA) test was used to compare the mean Control-Ct and miR-21 Ct values between three study sites. Student Paired *t*-test was used to compare the mean values of Ct between control and miR-21 in different sites. The Chi-square test was used to compare the miR-21 Ct interpretation between different study sites. The level of significance (*P*-value) was set at $P < 0.05$. All calculations were performed using the SPSS software (version 22.0. Released 2013. Armonk, NY, USA: IBM Corp).

RESULTS

MiR-21 expression profile was quantified and compared between three tumor sub-sites of the oral cavity such as buccal mucosa, tongue and GB sulcus and miR-21 was found to be significantly expressed. Out of 30 samples, 22 showed upregulation and 8 showed downregulation compared to the controls. Differential expression of miR-21 was measured using RT-PCR. The mean Ct of miR-21 was compared to U6 and the obtained difference value was used as quantitative data for the statistical comparison of study groups.

The comparison of miR-21 Ct interpretation between the study sites was done using the Chi-square test. Among the three groups, higher upregulation was noted in the buccal mucosa (90%), followed by GB sulcus (70%) and tongue (60%). It demonstrated no significant statistical difference at subsites ($P = 0.30$), as shown in Table 2 and Figure 1.

One-way ANOVA test was used to compare the mean miR-21 Ct values between the three study sites ($P = 0.40$), as shown in Table 3 and Figure 2.

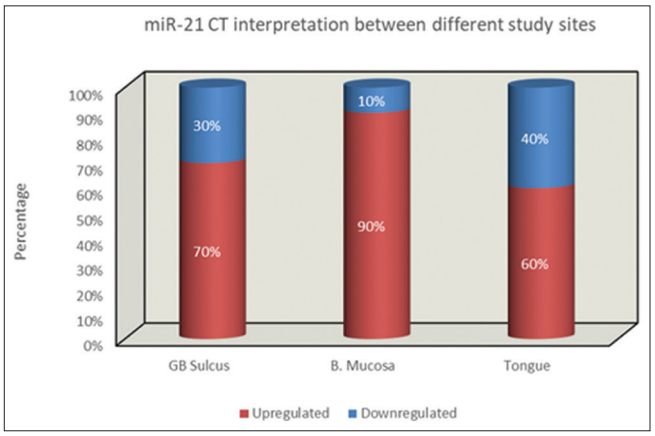

Figure 1: MiR-21 cycle threshold interpretation in sub-sites

Table 1: Primer sequences used in the study

Gene	Primer sequence (5'-3')
miR-21, forward	ACGTTGTGTAGCTTATCAGACTG
miR-21, reverse	AATGGTTGTTCTCCACACTCTC
U-6, forward	AATGGTTGTTCTCCACACTCTC
U-6, reverse	GGAACGCTTCACGAATTTG

Table 2: Comparison of miR-21 Ct interpretation in sub-sites

Comparison of the miR-21 Ct interpretation between different study sites using the Chi-square test				
Sites	**Upregulated, *n* (%)**	**Downregulated, *n* (%)**	**χ^2**	***P***
GB sulcus	7 (70)	3 (30)	2.386	0.30
Buccal mucosa	9 (90)	1 (10)		
Tongue	6 (60)	4 (40)		

GB: Gingivobuccal

Table 3: Comparison of mean values of miR-21 Ct values at sub-sites

Comparison of mean values of miR 21-Ct between three study sites using one-way ANOVA test						
Sites	***n***	**Mean**	**SD**	**Minimum**	**Maximum**	***P***
GB Sulcus	10	24.11	1.97	20.7	26.4	0.40
Buccal mucosa	10	24.49	1.86	21.4	26.2	
Tongue	10	25.15	1.20	22.6	26.7	

SD: Standard deviation, GB: Gingivobuccal

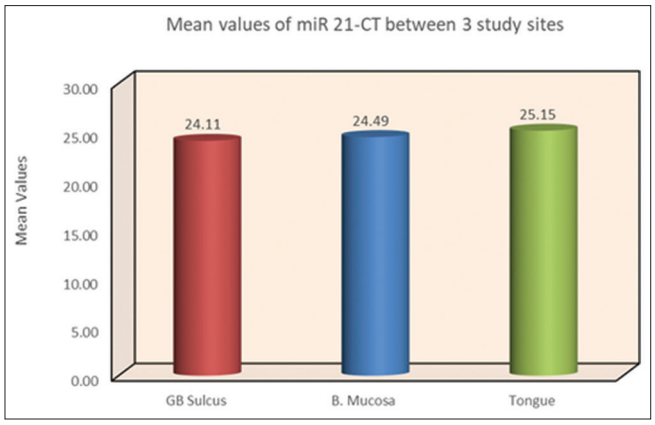

Figure 2: Mean values of miR-21 cycle threshold values at Sub-sites

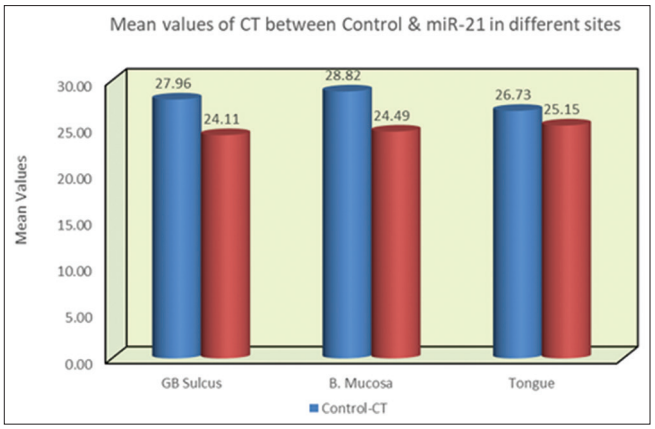

Figure 3: Cycle threshold values of sub-sites of oral squamous cell carcinoma

Table 4: Comparison of mean values of Ct between U6 and miR-21

Comparison of mean values of Ct between control and miR-21 in different sites using student paired *t*-test						
Groups	**CT**	***n***	**Mean**	**SD**	**Mean difference**	***P***
GB sulcus	Control-CT	10	27.96	3.29	3.85	0.01*
	miR-21 CT	10	24.11	1.97		
Buccal mucosa	Control-CT	10	28.82	2.97	4.33	0.002*
	miR-21 CT	10	24.49	1.86		
Tongue	Control-CT	10	26.73	3.20	1.58	0.25
	miR-21 CT	10	25.15	1.20		

*SD: Standard deviation, GB: Gingivobuccal, CT: Threshold Cycle

The comparison of mean values of Ct between control and miR-21 was done using student "*t*- test." miR-21 was significantly upregulated in the buccal mucosa ($P = 0.002$), followed by GB sulcus ($P = 0.01$) and tongue ($P = 0.25$), as depicted in Table 4 and Figure 3.

There is dearth of data for the comparison of our study results for the site specificity of miR-21 expression. However, sub-site comparison of miR-21 expression showed significantly altered expression in specific tumor sites.

DISCUSSION

MiRNAs play a significant role in controlling particular expression of genes. Hence, dysregulation (up and downregulation) of miRNA is expected to be existing in OSCC. It is suggested that miRNA alteration could initiate carcinogenesis.[3] miRNAs are hypothesized to be more stable in FFPE samples due to their small size and secondary structure.[8] miR-21 is considered as one of the first miRNAs to be detected in human genome and is found to be overexpressed or upregulated in different tumor types including head and neck SCC.[10]

miR-21 which functions as oncogene is found to serve as a diagnostic and prognostic marker for cancer therapy. The expression levels of miR-21 in OSCC are associated with a poor prognosis and multiple lines of evidence indicate the involvement of miR-21 in the aggressiveness of OSCC.[11]

A handful of studies exist in relation to the subsite specificity of miR-21 expression in OSCC. In the present study, we aimed to analyze the site specificity of miR-21 expression in three different sites of the oral cavity using RT-PCR.

In a comprehensive systematic review and meta-analysis, it was found that total of seven studies assessed miR-21 expression in head and neck cancer patients and all seven studies showed upregulation of miR-21. The pooled effect size estimate was found to be statistically significant ($P < 0.05$) and six studies showed that upregulated miR-21 expression leads to a lower probability of survival.[12]

Previous studies showed that different areas within oral cavity vary in their miRNA expression. miR-424 was studied in tongue SCC, and no significant difference was seen between gingival tumors or tumors of the floor of the mouth.[13]

A recent study showed that miR-21 was expressed in stromal cells of OSCC of the tongue and floor of the mouth suggesting that miR-21 expression reflects a pathological process in the stromal compartment and demonstrated that neoplastic progression is not solely determined by the cancer cells but also by stromal processes surrounding the tumor.[14]

From the analysis of 836 miRNAs in FFPE samples from tongue SCC, 54 miRNAs were identified to be differentially expressed. Among these, miR-21 was the second highest up regulated, and miR-203 was among the most downregulated miRNAs.[15]

Gombos *et al*. analyzed the expression alterations of miR-21 from the cancer field of clinically early-stage OSCC and compared them with those of the normal mucosa and showed significant overexpression of miR-21. The results underlined the role of miR-21 in OSCC.[16]

A study by Boldrup *et al*. have shown that miR-21 to be upregulated and miR-125b and miR-203 to be downregulated in tongue SCC compared with clinically normal tissue adjacent to tumors. miR-21 was not significantly altered in gingival tumors but significantly upregulated in both tongue and floor of the mouth tumors.[15]

miR-21 expression did not report any difference among tumors of oropharynx, oral cavity, larynx and hypopharynx. Sub-site analysis to determine if there were tissue-specific differences between sub-sites was performed, however none of these yielded any statistical significance.[7]

A clinicoepidemiological study involving 147 cases of SCC from the buccal mucosa and 94 cases from the tongue was undertaken to check if any difference existed in the cell cycle regulatory mechanism of the tumors by comparing immunohistochemically the expression of major cell cycle regulatory proteins in two sub-sites of oral cavity.[17]

In our study, we were able to quantify the miR-21 expression at three subsites of OSCC with significant upregulation and differential expression was also noted at subsites. The present study aimed to contribute for the investigation of miR-21 at subsites of OSCC, although it did not illustrate the statistical significance. The reason could be due to the weak expression of miR-21 and probably due to small sample size.

CONCLUSION

The etiology and clinical appearance of SCC involving various mucosae of the oral cavity differ. Morphological, genetic and epigenetic alterations may substantiate to the diversity of OSCC. It is therefore important to pay particular attention to the studies on subsite specificity of OSCC which may furnish the evidence for the genetic alterations and behavioral patterns pertaining to the specific site in the oral cavity. Studies on miRNAs are swiftly expanding and further studies on a large scale are required to prove the ability of miR-21 as a site-specific diagnostic marker in correlation with the genetic pathways.

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Conflicts of interest
There are no conflicts of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Sarode G, Sarode S C, Tupkari J, Patil S. Is oral squamous cell carcinoma unique in terms of intra-and inter-tumoral heterogeneity? Translational Research in Oral Oncology 2017;2:1-6.
2. Trivedi TI, Tankshali RA, Goswami JV, Shukla SN, Shah PM, Shah NG. Identification of site-specific prognostic biomarkers in patients with oral squamous cell carcinoma. Neoplasma 2011;58:217-26.
3. Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin Cancer Res 2008;14:2588-92.
4. Pérez-Sayáns M, Pilar GD, Barros-Angueira F, Suárez-Peñaranda JM, Fernández AC, Gándara-Rey JM, *et al*. Current trends in miRNAs and their relationship with oral squamous cell carcinoma. J Oral Pathol Med 2012;41:433-43.
5. Min A, Zhu C, Peng S, Rajthala S, Costea DE, Sapkota D. MicroRNAs as important players and biomarkers in oral carcinogenesis. Biomed Res Int 2015;2015:186904.
6. Barker EV, Cervigne NK, Reis PP, Goswami RS, Xu W, Weinreb I, *et al*. microRNA evaluation of unknown primary lesions in the head and neck. Mol Cancer 2009;8:127.
7. Chang SS, Jiang WW, Smith I, Poeta LM, Begum S, Glazer C, *et al*. MicroRNA alterations in head and neck squamous cell carcinoma. Int J Cancer 2008;123:2791-7.
8. Rentoft M, Fahlén J, Coates PJ, Laurell G, Sjöström B, Rydén P, *et al*. miRNA analysis of formalin-fixed squamous cell carcinomas of the tongue is affected by age of the samples. Int J Oncol 2011;38:61-9.
9. Luo J, Ning X, Wang H, Liang J, Lu X. MicroRNA-21 promotes axonal regeneration of peripheral nerve cells through regulating EPHA4 expression in schwann cells. Int J Clin Exp Med 2017;10:14203-12.
10. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 2004;10:1957-66.
11. Yu EH, Tu HF, Wu CH, Yang CC, Chang KW. MicroRNA-21 promotes perineural invasion and impacts survival in patients with oral carcinoma. J Chin Med Assoc 2017;80:383-8.
12. Kumarasamy C, Madhav MR, Sabarimurugan S, Krishnan S, Baxi S, Gupta A, *et al*. Prognostic value of miRNAs in head and neck cancers: A comprehensive systematic and meta-analysis. Cells 2019;8.
13. Boldrup L, Coates PJ, Laurell G, Wilms T, Fahraeus R, Nylander K. Downregulation of miRNA-424: A sign of field cancerisation in clinically normal tongue adjacent to squamous cell carcinoma. Br J Cancer 2015;112:1760-5.
14. Hedbäck N, Jensen DH, Specht L, Fiehn AM, Therkildsen MH, Friis-Hansen 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:e95193.
15. Boldrup L, Coates PJ, Wahlgren M, Laurell G, Nylander K. Subsite-based alterations in miR-21, miR-125b, and miR-203 in squamous cell carcinoma of the oral cavity and correlation to important target proteins. J Carcinog 2012;11:18.
16. Gombos K, Horváth R, Szele E, Juhász K, Gocze K, Somlai K, *et al*. miRNA expression profiles of oral squamous cell carcinomas. Anticancer Res 2013;33:1511-7.
17. Sathyan KM, Sailasree R, Jayasurya R, Lakshminarayanan K, Abraham T, Nalinakumari KR, *et al*. Carcinoma of tongue and the buccal mucosa represent different biological subentities of the oral carcinoma. J Cancer Res Clin Oncol 2006;132:601-9.