Original Article

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Oncology Section

Pre and Postoperative Serum miR-21 Expression Levels in Oral Cancer and it's Association with Clinical and Histological Parameters: An Observational Study

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ABSTRACT

Introduction: The patterns of microRNA (miRNA) expression have opened up new avenues in the search for prognostic biomarkers and potential therapeutic targets for various tumours. In oral carcinogenesis research, analysing microRNAs expressed in Oral Squamous Cell Carcinoma (OSCC) unveils a complex network of interest. miR-21 is known to be overexpressed in numerous solid tumours and is linked to the progression of malignancies in hepatocellular carcinomas, breast cancer, and colon carcinomas.

Aim: To determine the impact of surgical excision on serum miR-21 expression levels in OSCC cases and to establish correlations with clinicopathological parameters.

Materials and Methods: The study was an exploratory prospective observational study conducted with proper institutional ethical approval at the Cancer Research Institute (CRI), Himalayan Institute of Medical Sciences (HIMS), Jolly Grant, Dehradun, Uttarakhand, India. Fifty-six histologically confirmed OSCC cases were enrolled along with 25 healthy subjects as controls from May 2021 to June 2023. The relative fold expression change was calculated using the Livak method ($2^{-\Delta\Delta ct}$). Data analysis was performed using software such as Statistical Package for Social Sciences (SPSS) version 20.0 and MS Excel. Non parametric statistical tests such as Wilcoxon signed-rank test, Mann-Whitney test, and Kruskal-Wallis test were utilised.

Results: Out of 56 sample included, 28 exhibited high expression of miR-21 (p-value<0.001), while 27 cases showed downregulation postsurgery (p-value<0.001), with one sample showing the same level of expression. In cases where the time difference between pre and postsurgery samples was over 25 days, no significant change was observed (p-value=0.06), and similarly, in cases with a time difference below 25 days, no significant difference was noted (p-value=0.14). A significant negative correlation was found between presurgery serum Albumin to Globulin ratio (A:G ratio) and miR-21 expression (p-value <0.05), while no other parameters showed a significant correlation with miR-21.

Conclusion: miR-21 expression decreased significantly in some OSCC cases; however, in the majority of cases, it increased postsurgery. A significant correlation was observed between miR-21 expression and the serum A:G ratio.

Keywords: Clinicopathological parameters, microRNA, Perineural invasion, Tumour necrosis

INTRODUCTION

One of the most common malignancies in the world is OSCC. Because the majority of OSCC patients are detected in advanced stages of the disease, the 5-year survival rate is only around 50% [1]. Its prognosis has not improved, and its incidence has grown in the last few decades despite breakthroughs in biology and technology. Innovations in prognostic and diagnostic tools for the clinical setting are necessary because of the complexity and severity of OSCC [2]. By identifying the affected molecular pathways, biomarkers that might be used to anticipate a tumour's progress or facilitate an early diagnosis can be found. Small, non coding RNAs with a length of 18-25 nucleotides known as miRNAs, control post-transcriptional regulation of protein production [2].

Among the physiological processes in which miRNAs are engaged are apoptosis, angiogenesis, immune response, cell division, proliferation, and differentiation [3]. Certain miRNAs' dysregulation has been linked to the development and spread of cancer. Furthermore, studies have demonstrated that the disrupted cells release miRNAs into the bloodstream and other bodily fluids [4,5]. Deregulation of certain microRNA panels has been linked to the diagnostic and prognostic values of several malignancies, including oral cancer. The therapeutic usefulness of miRNA profiles in head and neck cancer research is limited by new difficulties. Presently published literature identifies several factors associated with these discrepancies: sample type (frozen tissue, cell lines embedded in paraffin, or formalin-fixed tissue); origin and localisation (oral cavity, pharynx, or larynx); platforms utilised; and lesion type (ranging from premalignant lesions to advanced lesions with metastasis) [6,7].

Several earlier investigations have noted that miR-21 is overexpressed in a large number of solid tumours; moreover, this overexpression has been linked to the advancement of malignancies in hepatocellular carcinomas, breast cancer, and colon carcinomas [4,8]. The majority of previous studies have focused on finding microRNA expression alterations in cancer cases and healthy controls, particularly emphasising tissues and plasma [2,9]. Additionally, a few studies have investigated miR-21 as a prognostic marker [10,11]. Therefore, the available information regarding serum miR-21 expression levels remains unclear in postoperative cases of OSCC, as well as its usefulness as a biomarker that can be non invasive or minimally invasive in nature. This study was conducted to evaluate the impact of surgical excision on the serum miR-21 in OSCC cases, along with its correlation with tumour microenvironment variables such as mitotic figures, presence of necrosis, nodal metastasis, tumour invasion status, and other factors collected through histopathological and other clinical parameters.

MATERIALS AND METHODS

This study was designed as an exploratory prospective observational study, conducted at CRI, HIMS, Jolly Grant, Dehradun, Uttarakhand, India from May 2021 to June 2023. The study was approved by

the Institutional Ethics Committee (IEC) with the reference number SRHU/HIMS/ETHICS/2021/29. All subjects who participated in this study filled an informed consent form.

Inclusion criteria: Fresh or newly diagnosed cases of OSCC were included in the study.

Exclusion criteria: Patients with recurrent malignancy. HIV and any other immunological disorder, patients with terminal stage of disease were excluded from the study.

In this study, convenient sampling was conducted. A total of 56 histologically confirmed OSCC cases were randomly selected and enrolled, along with 25 age- and sex-matched healthy subjects as controls to normalise miR-21. The following demographic factors were recorded for each patient: age, sex, alcohol, tobacco, and smoking status. Furthermore, the histopathological factors (tumour necrosis, perineural invasion, vascular invasion, mitotic figures, TNM status, lymph node status), biochemical parameters, and clinical parameters such as clinical staging (American Joint Committee on Cancer Staging, 8th edition, 2018) [12], serum A:G ratio, neutrophil-to-lymphocyte ratio, and platelet-to-lymphocyte ratio of all OSCC cases were collected during the study.

Approximately, 3-4 mL of pre- and postsurgery blood samples was collected in yellow cap Serum-Separating Tubes (SST) from the patients. The time gap between presurgery and postsurgery samples was typically around 2-5 weeks. The collected samples were first set aside for 20-30 minutes, then centrifuged (REMI) at 3000 rpm for 15 minutes at 4°C. The light yellow or yellow-colored serum was separated from the blood. The separated serum would be stored at -80°C for further processing.

RNA Isolation and cDNA Preparation: For RNA isolation, RNA-Xpress™ Reagent (Himedia MB-6017) is used according to the manufacturer's instructions. The Takara Primescript™ RT reagent kit (cat # RR037A) was utilised to reverse transcribe the separated RNA into cDNA in a final reaction volume of 20 µL using PCR (Veriti from Applied Biosystems). All reactions were performed with the following conditions: 10 µL of RNA, 4.5 µL buffer, 0.5 µL RT Enzyme, 1 µL miR-21 RT primer, 1 µL U6 RT primer, and 3 µL RNAase-free water. The RT-PCR conditions were 37°C for 15 minutes, 42°C for two minutes, and 85°C for five seconds, followed by holding at 4°C.

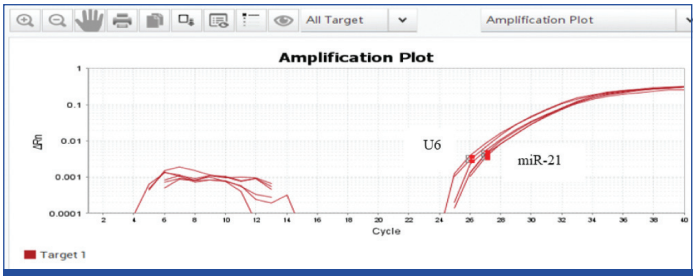
qPCR reaction: For qPCR, Takara TB Green® Premix Ex Taq™ (Tli RNase H Plus) (Cat #RR420A) was used according to the manufacturer's instructions. All the reactions were performed in triplicate. Each reaction was performed at a final volume of 10 µL containing 1 µL cDNA, 5 µL of TB Green premix (Ex TaqII), 0.5 µL human pre-miR-21 forward primer, 0.75 µL human pre-miR-21 reverse primer, 1 µL U6 forward primer, 0.75 µL U6 reverse primer, 0.2 µL ROX dye II, and 0.8 µL RNase-free water. The conditions for qPCR were as follows: first denaturation at 94°C for three minutes, then 40 cycles of 94°C for 15 seconds (denaturation), 60°C for 20 seconds (annealing), 70°C for 40 seconds (extension), and a final step at 94°C for one minute. The reactions were carried out in a Thermo Fisher lightcycler real-time PCR system. The relative fold expression change was calculated using the Livak method ($2^{-\Delta\Delta ct}$) [13]. The sigmoid curves showing the expression of U6 (internal control) or miR-21 (gene of interest) with Ct values shown below are from one of the samples of OSCC cases in the study [Table/Fig-1].

STATISTICAL ANALYSIS

All data were analysed using SPSS 20.0 and MS Excel. Depending on the normality of the data, the median (IQR) was used. Non parametric statistical tests such as Wilcoxon signed-rank test, Mann-Whitney test, and Kruskal-Wallis test were utilised. The level of significance considered in the study was p-value ≤0.05.

RESULTS

In this study, 56 OSCC cases were enrolled, with 47 cases involving men and nine cases involving women. The mean age in the study population was 49.1±1.3 years. No significant relationship was established between sex and miR-21 expression, while significantly higher miR-21 expression was observed in individuals aged over 50 years (p-value=0.02). A significant association between miR-21 expression and alcohol consumption (p-value <0.001) was observed, while the p-values for smokers and tobacco users were 0.07 and 0.139, respectively, which were non significant [Table/Fig-2].



[Table/Fig-1]: This graph is showing miR-21 and U6 gene expression of sample seen in Quantstudio software for qPCR.

Demographic parameter	n (%)	Median (IQR)	p-value
Sex			
Male	47 (83.9)	0.79 (3.4-0.64)	0.246
Female	9 (15.1)	0.27 (0.63-0.88)	
Age (years)			
≤50	28 (50)	0.12 (2.33-0.012)	0.02*
>50	28 (50)	0.78 (4.38-0.27)	
Tobacco chewer			
Yes	28 (50)	0.95 (4.04-0.23)	0.139
No	28 (50)	0.27 (2.67-0.30)	
Smoking			
Yes	17 (30.4)	1.28 (4.8-0.83)	0.07
No	39 (69.6)	0.28 (0.96-0.388)	
Alcohol consumption			
Yes	43 (76.8)	0.26 (1.28-0.23)	<0.001*
No	13 (23.2)	3.48 (10.34-1.17)	

[Table/Fig-2]: Presurgery serum miR-21 expression in demographic parameters of OSCC cases.
*: significant p-value

The change in serum miR-21 expression of oral cancer cases between pre- and post-tumour resection was calculated using the Wilcoxon-Signed Rank test, as the data were found to be non parametric. It was observed that the change in miR-21 expression was non significant (p-value=0.75) between presurgery and postsurgery in the serum samples of OSCC cases [Table/Fig-3]. However, out of 56 samples, 28 samples showed high expression of miR-21 postsurgery (p-value<0.001), while 27 samples showed down-regulated expression which was significant (p-value<0.001) postsurgery and one sample showed the same level of expression. The miR-21 expression in healthy controls was considered as a baseline.

Serum miR-21 expression and its correlation with clinical parameters such as tumour necrosis, perineural invasion, vascular invasion, mitotic figures, depth of invasion, invasion to adjacent site of the tumour, and clinical stage were also investigated in this study. The tests used to investigate these parameters were the Mann-Whitney test and Kruskal-Wallis test. The analysis of histological parameters showed no significant changes except for perineural invasion (p-value=0.05), indicating that miR-21 expression is affecting perineural invasion in OSCC cases [Table/Fig-4].

There was no significant relationship seen between clinical staging and miR-21 expression in OSCC cases, while the highest miR-21 expression was observed in cases with IVb staging [Table/Fig-5].

There were 18 patients in whom the difference between pre- and postsurgery samples was above 25 days, and no significant change

	No. of samples	Median (IQR)	p-value
Presurgery	56	0.37 (3.186-0.65)	0.75
Postsurgery	56	0.52 (2.57-0.110)	

	No. of cases which showed low level of miR-21 expression in postsurgery samples	Median (IQR)	p-value	No. of cases which showed high level of miR-21 expression in post-surgery samples	Median (IQR)	p-value
Presurgery	27	0.96 (3.9-0.20)	<0.001*	28	0.23 (1.56- 0.00)	<0.001*
Postsurgery	27	0.41 (1.2-0.10)		28	0.54 (14.6- 0.008)	

[Table/Fig-3]: Comparison between total pre- and postsurgery serum miR-21 expression of OSCC cases.
1 OSCC sample has same level of serum miR-21 expression in pre and postsurgery conditions; *: significant p-value

Clinical and histological parameter	n (%)	Median (IQR)	p-value
Tumour invasion to adjacent site			
Yes	18 (32.1)	0.43 (3.07-0.132)	0.90
No	38 (67.9)	0.369 (3.29-0.17)	
Tumour necrosis			
Yes	40 (71.4)	0.60 (3.92-0.839)	0.65
No	16 (28.6)	0.29 (2.30-0.216)	
Perineural invasion			
Yes	18 (32.1)	0.87 (4.67-0.457)	0.05*
No	38 (67.9)	0.30 (2.82-0.745)	
Vascular invasion			
Yes	32 (57.1)	0.20 (0.93-0.20)	0.79
No	24 (42.9)	1.83 (4.81-0.27)	
Mitotic figure			
≤2	34 (60.7)	0.62 (3.48-0.67)	0.420
>2	22 (39.3)	0.28 (2.4-0.47)	
TNM, Tumour			
T1-T2 (Early stage)	31 (55.3)	0.79 (3.4-0.14)	0.269
T3-T4 (Late stage)	25 (44.7)	0.20 (2.8-0.25)	
Nodes			
Positive	32 (57.1)	0.30 (3.26-0.54)	0.486
Negative	24 (42.9)	0.78 (3.16-0.10)	
Lymph nodes with mets			
Negative	35 (62.5)	0.30 (2.61-0.05)	0.486
Positive	21 (37.5)	0.62 (3.57-0.06)	

[Table/Fig-4]: Presurgery serum miR-21 expression level with clinical and histo-pathological data.
*: significant p-value

Clinical stage	N	Median (IQR)	p-value
I	7	0.96 (14.6-0.21)	p=0.131
II	16	0.28 (3.025-0.17)	
III	11	0.31 (2.61-0.60)	
Iva	16	0.125 (1.20-0.100)	
IVb	6	2.81 (26.4-0.36)	

[Table/Fig-5]: Presurgery serum miR-21 expression level in clinical staging of OSCC cases.

was found in those patients (p-value=0.06), while in 38 patients in whom the difference was below 25 days, no significant value was observed (p-value=0.14) [Table/Fig-6].

miR-21 and other parameters: To find out the correlation between presurgery miR-21 expression level and the duration of symptoms (in days), depth of invasion (in mm), mitotic figures (per high-power field), serum A:G ratio, Neutrophil to Lymphocyte Ratio (NLR) and Platelet to Lymphocyte Ratio (PLR), total protein (serum), etc., the Spearman's rho test was used. As the correlation between presurgery serum A:G ratio and miR-21 was significant (p-value=0.053), the albumin-to-globulin ratio was negatively correlated with miR-21 expression in serum; that is, with the increase in miR-21 expression, the A/G ratio will decrease,

Difference between pre- and postsurgery sample collection	N	Condition	Median (IQR)	p-value
Below 25 days	38	Presurgery	0.295 (3.01-0.60)	0.14
		Postsurgery	0.40 (0.06-13.24)	
After 25 days	18	Presurgery	0.79 (3.9-0.155)	0.06
		Postsurgery	0.615 (1.389-0.199)	

[Table/Fig-6]: Serum sample collection time (pre- and postsurgery sample) and miR-21 expression level in OSCC.

as the correlation coefficient (r) is -0.325. No other parameter showed any significant correlation with miR-21 [Table/Fig-7].

Category	Mean±SD	N	Correlation coefficient	p-value
Duration of symptoms (in days)	5.1±4.9	56	0.22	0.10
Mitotic figure (per HPF)	2.09±0.9	56	0.171	0.20
Depth of invasion (in mm)	10.64±7.9	56	-0.020	0.88
A:G ratio (serum)	1.3±0.28	36	-0.325	0.05*
NLR	3.6±4.0	40	-0.046	0.77
PLR	9.5±6.9	40	-0.040	0.80
Total Protein (serum)	7.3±0.6	34	0.147	0.40

[Table/Fig-7]: Presurgery miR-21 expression level and other parameters.
*: Significant p-value

DISCUSSION

In this study, the expression levels of serum miR-21 were examined in fresh cases of OSCC and after a few weeks of the tumour resection of oral cancer. A total of 56 histologically confirmed oral cancer cases were enrolled, of which 47 cases were men and nine cases were women. The study found that 27 OSCC cases (p-value≤0.001) showed low expression of miR-21 in the postoperative condition, while 28 OSCC cases (p-value≤0.001) showed high expression. Eighteen OSCC cases in which the presurgery and postsurgery difference was more than 25 days showed a slight change in the median value of miR-21 expression (p-value=0.06), but 38 OSCC cases in which the difference was less than 25 days also showed no significant change (p-value=0.14). It has been found that miR-21 is involved in the inflammation of wounds and their healing. miRNAs are involved in modulating the inflammatory response, promoting angiogenesis, and facilitating re-epithelialisation, which are essential phases for effective wound repair [14]. This could be one of the reasons why higher expression was found in those cases where the difference was less than 25 days. Most studies have found high miR-21 expression in oral cancer tissues and compared it with healthy individuals or precancerous conditions [2,9]. Additionally, in this study, there were no significant changes in miR-21 expression between pre- and postsurgery OSCC cases. A study conducted by Hsu CM et al., revealed a noteworthy alteration in the plasma samples obtained before and after surgery of OSCC, but it also showed that the expression of miR-21 remained elevated in patients who did not recover [10]. Another longitudinal investigation on plasma miR-21 in Head and Neck Squamous Cell Carcinoma (HNSCC) revealed that in the event of no recurrence, the expression level of plasma miR-21 was significantly lower two months after therapy. However, in the instance of 10 patients who experienced

recurrences throughout the follow-up period, plasma miR-21 did not decrease following treatment [11].

A study performed by Singh P et al., on oral cancer showed a significant change in miR-21 expression levels in oral cancer and pre-malignant lesions in the oral cavity. Additionally, high expression of miR-21 was detected from stages I-IV, which was related to different stages from early to later stages [15]. This study revealed no association between the clinical state and miR-21 expression in patients with OSCC. However, the highest median miR-21 expression was seen in stage IV OSCC cases.

In this study, other parameters such as perineural invasion (p-value=0.05) and A/G ratio (p-value=0.05) produced significant values with up-regulated serum miR-21 levels (presurgery). Perineural invasion, a pathological characteristic, has the potential to influence the prognosis, particularly in cases of OSCC, by affecting the growth of cancer cells. According to the study by Yu EH et al., miR-21 may encourage a cancer cell's invasion and dissemination within a nerve bundle [16]. This study also showed a positive association between perineural invasion and miR-21 expression. Furthermore, increasing evidence shows that miR-21 contributes to the perineural invasion of certain non neural origin tumours. It was also demonstrated that miR-21 might promote perineural invasion of oral carcinoma through inhibiting the Phosphate and Tensin Homolog Gene (PTEN) [17].

The A:G is a significant biomarker used to monitor inflammation, nutritional status, and predict various health outcomes [18]. Up-regulation of miR-21 has been associated with lung cancer, stomach cancer, pancreatic cancer, breast cancer, glioblastoma, neuroendocrine tumours, colon cancer, and prostate cancer [5,7-9]. Numerous investigations have demonstrated the significance of miR-21 in the diagnosis and assessment of tumours. In another study on oral cancer patients, a poor prognosis and lymph node metastases in addition to high expression of miR-21 were recorded [19]. However, in this study, no significant association was established.

Limitation(s)

The small sample size and early postsurgery sample collection period are major limitations of this study. This suggests that to determine the impact of variations in miR-21 expression in serum, a larger sample size is required and an extended follow-up period of patients, which can help maximise the biomarker's predictive potential.

CONCLUSION(S)

Although miR-21 expression was significantly reduced in a few OSCC patients, it remained high in the majority of cases in the postsurgery state. Despite the strong correlation established Between miR-21 and perineural invasion in tissue and serum A:G ratio, further research is still required to fully understand the relationship between microRNA and other clinical and histological markers. Therefore, more studies are needed to consider miR-21 expression in OSCC as a predictive biomarker. It is important to note that research on postsurgery miR-21 expression levels in oral cancer is still evolving, and postsurgery miRNA expression is a promising area of research with the potential to improve the management of oral cancer. Large datasets may be needed to fully exploit these short RNAs as a therapeutic diagnostic and prognostic tool for OSCC.

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