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ORIGINAL ARTICLE

MicroRNA-21 Boon For Early Diagnosis And Treatment Modifications In Oral Cancers Of North Indian Population

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is a major type of oral cancer with significant global health implications. MicroRNAs (miRNAs) play crucial roles in gene regulation and have been implicated in OSCC development, particularly miR-21, which has shown oncogenic properties. Understanding miRNA expression profiles and their association with OSCC progression can provide valuable insights for diagnosis and therapeutic approaches.

Methods:RNA extraction was performed using a ……. Nucleic Acid Extraction Kit followed by cDNA synthesis with a Thermo Fisher Revert Aid cDNA Synthesis Kit. MiR-21 expression was analyzed using real-time PCR with SYBR Green Quantitative PCR reagent kit. Statistical analyses were conducted to evaluate associations between miRNA-21 levels and demographic variables, disease stages, and clinical features.

Results:The study analyzed miRNA-21 expression in patients with oral cancer, including SCC and Adenocarcinoma. It investigated the relationship between miRNA-21 levels and demographic variables, clinical features, and disease stages. The results indicated that miRNA-21 expression increased with advancing disease stages and poorly differentiated lesions. However, age, sex, and site did not show significant associations with miRNA-21 levels.

Conclusion:In conclusion, miRNA-21 shows potential as a diagnostic and prognostic biomarker in OSCC and may hold promise as a therapeutic target for suppressing tumor growth. However, further research is needed to better understand its association with age, sex, and site distribution in OSCC.

Keywords: miRNA-21, oral cancer, SCC, Adenocarcinoma, disease stage, grade of lesion

I. BACKGROUND

Oral squamous cell carcinoma (OSCC) is a prominent contributor, accounting for 84-97% of all oral cancer cases. This type of cancer often arises from potentially malignant lesions or normal epithelial linings. OSCC manifests as unfamiliar growths or sores in various mouth parts, including the lips, cheeks, sinuses, tongue, hard and soft palate, and oropharynx. Globally, oral cancer ranks sixth among all cancer types, with India bearing the highest burden, accounting for one-third of global cases. The prevalence of oral cancer poses significant health challenges, particularly for countries undergoing economic transition. In India, there are approximately 77,000 new cases and 52,000 deaths reported annually, making up a

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considerable portion of global incidences [1].The rising incidence of oral cancer is a major concern for public health, particularly in India, where it is one of the most prevalent cancer types [2]. Compared to Western countries, India faces a significantly higher burden of oral cancer, with approximately 70% of cases being diagnosed in advanced stages (Stage III-IV) according to the American Joint Committee on Cancer [3]. Late detection leads to low chances of cure, resulting in five-year survival rates of only around 20% [3]. Additionally, certain conditions known as potentially malignant disorders (PMDs), including inflammatory oral submucosa, fibrosis, erythroplakia, leukoplakia, candidal leukoplakia, dyskeratosis congenita, and lichen planus, serve as idicators of the preclinical phase of oral cancer [4].Oral cancer risk factors include the use of tobacco, both in smoking and smokeless forms, betel-quid chewing, excessive alcohol consumption, poor oral hygiene, and a nutrient-deficient diet. Additionally, viral infections such as human papillomavirus (HPV) can also contribute to the development of oral cancer. Lack of awareness, exposure to harsh environmental conditions, and behavioral risk factors are key factors influencing the variation in oral cancer incidence worldwide.

MicroRNAs (miRNAs) are a group of small, naturally occurring non-coding RNA molecules that play a crucial role in controlling various biological processes by modulating gene expression. When these miRNAs are disrupted, they enable tumor cells to acquire tumor-promoting capabilities, such as enhanced signaling for cell proliferation, evasion of growth-inhibitory signals, resistance to programmed cell death, increased ability to invade surrounding tissues and metastasize, and the stimulation of new blood vessel formation (angiogenesis) [5].

The human miR-21 gene consists of 3433 nucleotides and is situated in a non-coding region on chromosome 17q23.2 [6]. This gene has two transcription sites, namely T1 (minor) and T2 (primary). Within the nucleus, RNA pol II enzyme facilitates the transcription of primary miR-21, a larger RNA molecule of approximately 3.5 kb. Subsequently, this primary miR-21 is transported to the cytoplasm, where polymerase III releases the 22-nucleotide mature miR-21. This mature miR-21 is then incorporated into the miRNA-RISC complex, where it undergoes degradation. In tumor cells, alterations in the regulation of miR-21 may occur due to changes in its expression, transcription, transport, binding with RISC, and degradation mechanisms [7,8].

The expression patterns of miRNAs reflect the tissue's developmental origin and are primarily specific to the head and neck region. This specificity may be attributed to embryological events that lead to the distinct anatomical sites, each having unique miRNA profiles [9]. Among these miRNAs, miR-21 has been extensively studied as a biomarker in OSCC. Experimental evidence indicates that miR-21 inhibits multiple tumor suppressor targets, including phosphatase and tensin homolog deleted on chromosome 10, Tropomyosin-1, and programmed cell death-4 [10]. In OSCC, miR-21 is known to be overexpressed and plays an oncogenic role by promoting cell proliferation, invasion, antiapoptosis, and chemoresistance, as demonstrated by numerous in vivo and in vitro experiments [11].

The aim of this study is to investigate the expression and role of miR-21 in OSCC and its association with tumor progression, cell proliferation, invasion, antiapoptosis, and chemo resistance.

II. MATERIAL AND METHODS

Subject'senrollment

A total of 70 biopsy proven cases of OSCC were recruited via purposive sampling from ENT, radiation, pathology, oncology departmentEra's Lucknow Medical College & Hospital.Prior to commencing the study, ethical approval was obtained from the Ethical Review Committee of Era Medical College and Hospital. This ensured that all research activities adhered to the highest ethical standards and were conducted with due respect for the participants'

rights and well-being. To safeguard the participants' rights and ensure their informed participation, written informed consent was obtained from all individuals who volunteered to participate in the study. The informed consent process emphasized the voluntary nature of their participation and provided a clear understanding of the study's objectives and procedures. The enrollment of participants with proper ethical considerations and adherence to inclusion criteria aimed to establish a comprehensive and reliable investigation into the expression levels of miRNA-21 in oral cancer.

Patient's characteristics and sample collection

Participants who had undergone biopsy or histological examination followed by confirmed diagnosis of oral cancer, specifically SCCor Adenocarcinoma were included the study. 03ml peripheral venous whole blood samplewas also collected from each subject for RNA extraction and subsequent miR-21 expression analysis. Comprehensive demographic and clinical information, including tumor stage, tumor grade, site of cancer, and metastasis status was collected from through hospital medical records. Participants with oral cancer subtypes other than SCC or Adenocarcinoma were excluded to maintain a homogenous study population.Participants who have received treatment for oral cancer before the study were excluded, as previous treatments may alter miRNA expression levels.Participants with pre-existing medical conditions that might impact miRNA expression or oral cancer progression were also excluded. Pregnant individuals were also excluded from the study to avoid potential confounding effects.

Real-Time Polymerase Chain Reaction (RT-PCR)

RNA Extraction

RNA extraction was performed on blood samples within 12-18 hours of collection to ensure minimal RNA degradation. Theblood sample was used to extract total RNA from the blood samples following the kit's protocol. This extraction method is designed to obtain high-quality RNA for downstream analysis.

cDNA Synthesis

Complementary DNA (cDNA) synthesis was carried out using the cDNA Synthesis Kit. For cDNA preparation, specific components were added to the eluted miRNA, including MMLV RT 4 µl, dNTPs 2 µl, DTT 0.5 µl, Oligo dT primer 1µl, reverted RT 1 µl, RNAse inhibitor 1 µl, distilled H2O 3.25 µl and eluted miRNA 4 µl to prepare a final volume of 17 µl.The cDNA synthesis was performed according to the kit's protocol, involving incubation at 42°C for 50 minutes, followed by 70°C for 10 minutes, and finally, storage at 4°C. The cDNA samples were stored at -80°C until further analysis.

MiR-21 Expression

The expression levels of miR-21 were analyzed using the previously reported primers by Wei et al. Specific primers for miR-21were used for sequencing. To assess the integrity and successful cDNA synthesis of miRNA, the expression of the GAPDH gene was evaluated as per the Thermo Scientific protocol. Samples expressing a 496 kb band of GAPDH were selected for miR-21 analysis. Additionally, the amplification of the internal control miR-16 provided further validation of good-quality miRNA extraction. For data normalization, miR-16 was used as the endogenous control.

Real-time PCR was performed using the SYBR Green Quantitative PCR Reagent Kit on the BioRad CFX96 analyzer. Each sample was analyzed in duplicate, and negative controls were included for accurate quantification. The 13 µl PCR volume for amplification included 2µl of cDNA, 6.5 µl of SYBR Green PCR Master Mix, 1.5 µl of primers and 3µl of H2O.The PCR program involved initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 62°C for 35 seconds. Fluorescence acquisition was performed during the annealing/extension step, and Rox was used as background noise in the PCR program.The expression levels of miR-21 were calculated

using the 2–ΔΔ Ct method, which allows for the relative quantification of miRNA expression levels normalized to the internal control miR-16. This method provides a quantitative assessment of miR-21 expression in the blood samples, allowing for comparisons between different groups and conditions.Overall, this study followed standardized protocols for RNA extraction, cDNA synthesis, and miR-21 expression analysis to ensure reliable and accurate results for investigating miR-21 expression levels in oral cancer.

Statistical analysis

Descriptive statistics were used to summarize and present the demographic distribution of subjects, including age, sex, and site of cancer, as well as the distribution of cases according to clinical findings, such as stage of disease, grade of lesion, and presence of metastasis. Mean values, standard deviations, percentages, and total counts were provided to describe the characteristics of the study population. The chi-square value and p-value were calculated to determine the significance of these associations. The t-test was used to compare the mean values of miRNA-21 expression (Delta Ct and Relative Fold Rise) between different groups, such as males and females or SCC and Adenocarcinoma cases. ANOVA was used to examine the relationship between miRNA-21 expression levels and different stages of disease and grades of lesion. Logistic regression analysis was employed to examine the relationship between miRNA-21 changes (Delta Ct and Relative Fold Rise) and disease stages. Ordinal regression analysis was used to predict the disease stage based on miRNA-21 levels (Delta Ct and Relative Fold Rise). The Nagelkerke R2 values were reported to indicate the proportion of variance explained by the logistic and ordinal regression models, providing information about the predictive power of the models.

III. RESULTS

Demographic Distribution of Subjects

The provided table illustrates the distribution of variables, including SCC (Squamous Cell Carcinoma) and Adenocarcinoma, along with their respective totals. The variables analyzed in the table are age, sex, and site. In terms of age, there were notable differences observed among the age groups. Individuals aged 30 to 39 years exhibited 4 cases of SCC (6.3%), with no instances of Adenocarcinoma (0.0%). This age group accounted for a total of 4 cases (5.7%). Among individuals aged 40 to 49 years, there were 12 cases of SCC (18.8%) and 1 case of Adenocarcinoma (16.7%). This age group accounted for a total of 13 cases (18.6%). The age group of 50 to 59 years exhibited 36 cases of SCC (56.3%) and 3 cases of Adenocarcinoma (50.0%), resulting in a total of 39 cases (55.7%). In the age group of 60 to 70 years, there were 12 cases of SCC (18.8%) and 2 cases of Adenocarcinoma (33.3%), resulting in a total of 14 cases (20.0%). However, statistical analysis indicated that the association between age and the occurrence of SCC and Adenocarcinoma was not significant, as indicated by a chi-square value of 1.01 and a p-value of 0.799. With regard to the sex variable, males exhibited a higher incidence of SCC, with 54 cases (84.4%), compared to females who had 10 cases (15.6%). Similarly, all cases of Adenocarcinoma were found in males (6 cases, 100.0%). However, the statistical analysis did not reveal a significant association between sex and the occurrence of SCC and Adenocarcinoma, as indicated by a chi-square value of 1.09 and a p-value of 0.296. In terms of site, the distribution of SCC and Adenocarcinoma cases across different sites showed some variations. The highest prevalence of SCC and Adenocarcinoma was observed in the buccal mucosa, with 32 cases of SCC (50.0%) and 3 cases of Adenocarcinoma (50.0%), resulting in a total of 35 cases (50.0%). Other sites, including the gingiva, lips, and tongue, exhibited varying proportions of SCC and Adenocarcinoma cases. The distribution of cases across these sites did not reach statistical significance, emphasizing the need for further analysis. However, statistical analysis did not

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establish a significant association between site and the occurrence of SCC and Adenocarcinoma, as indicated by a chi-square value of 1.40 and a p-value of 0.706.

Table 1: Demographic Distribution of Subjects

cc		SCC		Adenocarcinoma		Total		chi sq	p-value
		No.	%	No.	%	No.	%		
Age	30 - 39 yr	4	6.3%	0	0.0%	4	5.7%	1.01	0.799
	40 - 49 yr	12	18.8%	1	16.7%	13	18.6%		
	50 - 59 yr	36	56.3%	3	50.0%	39	55.7%		
	60 - 70 yr	12	18.8%	2	33.3%	14	20.0%		
sex	Male	54	84.4%	6	100.0%	60	85.7%	1.09	0.296
	Female	10	15.6%	0	0.0%	10	14.3%		
site	buccal mucosa	32	50.0%	3	50.0%	35	50.0%	1.40	0.706
	gingiva	18	28.1%	2	33.3%	20	28.6%		
	lips	9	14.1%	0	0.0%	9	12.9%		
	tongue	5	7.8%	1	16.7%	6	8.6%		

Distribution of Cases according to Clinical Findings

The provided table presents the results of the analysis of variables including SCC and Adenocarcinoma. The variables examined in the table are the stage of disease, grade of lesion, and the presence of metastasis. In terms of the stage of disease, the distribution of SCC and Adenocarcinoma cases across different stages showed the following results. For Stage I, there were 47 cases of SCC (73.4%) and 5 cases of Adenocarcinoma (83.3%), resulting in a total of 52 cases (74.3%). In Stage II, there were 8 cases of SCC (12.5%) and no cases of Adenocarcinoma (0.0%), resulting in a total of 8 cases (11.4%). For Stage III, there were 7 cases of SCC (10.9%) and 1 case of Adenocarcinoma (16.7%), making a total of 8 cases (11.4%). In Stage IV, there were 2 cases of SCC (3.1%) and no cases of Adenocarcinoma (0.0%), resulting in a total of 2 cases (2.9%). The chi-square value for this stage is 1.17, with a p-value of 0.761, indicating that there is no significant association between the stage of disease and the occurrence of SCC and Adenocarcinoma. Regarding the grade of lesion, the distribution of SCC and Adenocarcinoma cases based on lesion differentiation exhibited the following outcomes. Among well-differentiated lesions, there were 51 cases of SCC (79.7%) and 5 cases of Adenocarcinoma (83.3%), resulting in a total of 56 cases (80.0%). Among moderately differentiated lesions, there were 11 cases of SCC (17.2%) and 1 case of Adenocarcinoma (16.7%), making a total of 12 cases (17.1%).For poorly differentiated lesions, there were 2 cases of SCC (3.1%) and no cases of Adenocarcinoma (0.0%), resulting in a total of 2 cases (2.9%). The chi-square value for this grade is 0.20, with a p-value of 0.906, indicating no significant association between the grade of lesion and the occurrence of SCC and Adenocarcinoma. Regarding the presence of metastasis, the distribution of SCC and Adenocarcinoma cases indicated the following outcomes. Among cases without metastasis, there were 54 cases of SCC (84.4%) and 6 cases of Adenocarcinoma (100.0%), resulting in a total of 60 cases (85.7%). The chi-square value for this variable is 1.09, with a p-value of 0.296, suggesting no significant association between the presence of metastasis and the occurrence of SCC and Adenocarcinoma.

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Table 2: Distribution of Cases according to Clinical Findings

Variable		SCC		Adenocarcinoma		Total		chi sq	p-value
		No.	%	No.	%	No.	%		
Stage of Disease	I	47	73.4%	5	83.3%	52	74.3%	1.17	0.761
	II	8	12.5%	0	0.0%	8	11.4%		
	III	7	10.9%	1	16.7%	8	11.4%		
	IV	2	3.1%	0	0.0%	2	2.9%		
Grade of Lesion	Well differentiated	51	79.7%	5	83.3%	56	80.0%	0.20	0.906
	Moderately differentiated	11	17.2%	1	16.7%	12	17.1%		
	Poorly differentiated	2	3.1%	0	0.0%	2	2.9%		
Metastatis	No	54	84.4%	6	100.0%	60	85.7%	1.09	0.296
	Yes	10	15.6%	0	0.0%	10	14.3%		

Association of Demographic Variables with miRNA-21 Level

The table presents the results of the analysis of variables related to Delta Ct - mi-21 RNA and Relative Fold Rise mi-21 RNA, including their means and standard deviations. The variables examined in the table are age, sex, and group. For the age variable, the mean Delta Ct - mi-21 RNA values showed a slight increase from 30-39 years (9.33) to 60-70 years (11.52). However, the differences in mean values were not statistically significant, as indicated by an F-value of 1.05 and a p-value of 0.377. The Relative Fold Rise mi-21 RNA values displayed a more substantial increase with age, particularly from 50-59 years (22.99) to 60-70 years (33.84). Nevertheless, the statistical analysis did not reveal a significant association between age and Relative Fold Rise mi-21 RNA, with an F-value of 2.08 and a p-value of 0.111.In terms of sex, males exhibited a lower mean Delta Ct - mi-21 RNA (10.05) compared to females (12.25). However, the difference in means was not statistically significant, as indicated by a t-value of 1.83 and a p-value of 0.071. Similarly, the mean values of Relative Fold Rise mi-21 RNA were higher in females (41.30) compared to males (17.41), but the statistical analysis did not establish a significant association, with a t-value of 1.47 and a p-value of 0.172. Regarding the group variable, the mean Delta Ct - mi-21 RNA values were slightly higher in the SCC group (10.45) compared to the Adenocarcinoma group (9.48). However, the difference in means was not statistically significant, as indicated by a t-value of 0.64 and a p-value of 0.528. Similarly, the mean values of Relative Fold Rise mi-21 RNA were higher in the SCC group (22.05) compared to the Adenocarcinoma group (7.77), but the statistical analysis did not establish a significant association, with a t-value of 0.99 and a p-value of 0.325.

Table 3: Association of Demographic Variables with miRNA-21 Level

Variable	Delta Ct - mi-21 RNA	Relative Fold Rise mi-21 RNA

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		Mean	SD	Mean	SD
Age	30 - 39 yr	9.33	0.01	1.48	0.01
	40 - 49 yr	9.23	2.71	6.28	9.38
	50 - 59 yr	10.43	3.81	22.99	38.95
	60 - 70 yr	11.52	3.91	33.84	31.79
	significance	F=1.05, p=0.377		F=2.08, p=0.111	
Sex	Male	10.05	3.61	17.41	29.48
	Female	12.25	2.77	41.30	49.99
	significance	t=1.83, p=0.071		t=1.47, p=0.172	
Group	SCC	10.45	3.62	22.05	34.85
	Adenocarcinoma	9.48	2.99	7.77	14.17
	significance	t=0.64, p=0.528		t=0.99, p=0.325	

Association of Clinical Features of Disease with miRNA-21 Level

The provided table displays the results of the analysis of variables related to Delta Ct and Relative Fold Rise, including their means and standard deviations. The variables examined in the table are the stage of disease, grade of lesion, and the presence of metastasis. For the stage of disease variable, the mean Delta Ct values showed a gradual increase with advancing stages. Stage I had the lowest mean Delta Ct (8.96), followed by Stage II (13.75), Stage III (14.72), and Stage IV (15.82). The differences in mean values were found to be statistically significant, with an F-value of 18.89 and a p-value less than 0.001. Similarly, the mean values of Relative Fold Rise increased substantially with advancing stages, ranging from 5.85 in Stage I to 128.33 in Stage IV. The statistical analysis confirmed a significant association between the stage of disease and Relative Fold Rise, with an F-value of 57.32 and a p-value less than 0.001. Regarding the grade of lesion variable, the mean Delta Ct values displayed an increasing trend from well-differentiated lesions (9.36) to moderately differentiated lesions (14.16) and poorly differentiated lesions (15.82). The differences in mean values were found to be statistically significant, with an F-value of 16.51 and a p-value less than 0.001. Similarly, the mean values of Relative Fold Rise also increased progressively from well-differentiated lesions (10.39) to moderately differentiated lesions (51.63) and poorly differentiated lesions (128.33). The statistical analysis revealed a significant association between the grade of lesion and Relative Fold Rise, with an F-value of 35.75 and a p-value less than 0.001. Regarding the presence of metastasis variable, the mean Delta Ct values were lower in cases without metastasis (9.54) compared to cases with metastasis (15.34). The difference in means was found to be statistically significant, with a t-value of 5.77 and a p-value less than 0.001. Similarly, the mean values of Relative Fold Rise were higher in cases with metastasis (94.65) compared to cases without metastasis (8.52). The statistical analysis confirmed a significant association between the presence of metastasis and Relative Fold Rise, with a t-value of 16.94 and a p-value less than 0.001. In summary, the presented table provides insights into the mean values and standard deviations of Delta Ct and Relative Fold Rise based on the stage of disease, grade of lesion, and the presence of metastasis. The statistical analysis indicates significant associations between these variables and the measured values. These findings suggest that the stage of disease, grade of lesion, and presence of metastasis may influence the levels of Delta Ct and Relative Fold Rise, providing valuable information for further research and clinical considerations.

Table 4: Association of Clinical Features of Disease with miRNA-21 Level

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Variable		Delta Ct		Relative Fold Rise	
		Mean	SD	Mean	SD
Stage of Disease	I	8.96	2.98	5.85	11.66
	II	13.75	1.47	44.30	31.40
	III	14.72	0.77	67.81	33.74
	IV	15.82	0.04	128.33	0.04
	significance	F=18.89, p<0.001		F=57.32, p<0.001	
Grade of Lesion	Well differentiated	9.36	3.23	10.39	20.77
	Moderately differentiated	14.16	1.05	51.63	36.09
	Poorly differentiated	15.82	0.04	128.33	0.04
	significance	F=16.51, p<0.001		F=35.75, p<0.001	
Metastasis	No	9.54	3.16	8.52	13.56
	Yes	15.34	0.34	94.65	21.64
	significance	t=5.77, p<0.001		t=16.94, p<0.001	

Logistic Regression Analysis Showing Relationship of miRNA-21 changes with Disease Stages

The prediction equations of miRNA-21 changes are :

delta Ct = 8.96 + 4.79(stage II) +5.76(stage III) + 6.86(Stage IV)

relative FR = 5.85 + 38.45(stage II) +61.95(stage III) + 122.47(Stage IV)

The logistic regression analysis was conducted to examine the relationship between miRNA-21 changes and disease stages. The dependent variables were Delta Ct and Relative Fold Rise, while the disease stages (Stage II, Stage III, and Stage IV) were the independent variables.

For the Delta Ct model, the intercept value was 8.96, indicating the expected log-odds of the outcome (miRNA-21 changes) when the disease stage is at Stage I. The intercept had a significant effect on the outcome, with a t-value of 24.17 and a p-value of 0.000. The effect size (B) for the intercept was 0.899, implying a large effect. The R2 value for this model was 0.462, indicating that 46.2% of the variance in the outcome could be explained by the model. When comparing different stages to the reference category (Stage I), all stages (Stage II, Stage III, and Stage IV) showed significant effects on miRNA-21 changes, with positive effect sizes of 4.79, 5.76, and 6.86, respectively. These effect sizes represent the increase in the log-odds of the outcome for each unit change in the respective disease stage.

For the Relative Fold Rise model, the intercept value was 5.85, indicating the expected log-odds of the outcome when the disease stage is at Stage I. The intercept had a significant effect on the outcome, with a t-value of 2.32 and a p-value of 0.023. The effect size (B) for the intercept was 0.075, indicating a small effect. The R2 value for this model was 0.723, suggesting that 72.3% of the variance in the outcome could be explained by the model. Similar to the Delta Ct model, all stages (Stage II, Stage III, and Stage IV) had significant effects on miRNA-21 changes. The effect sizes for these stages were larger compared to the Delta Ct model, with values of 38.45, 61.95, and 122.47, respectively. These effect sizes represent the increase in the log-odds of the outcome for each unit change in the respective disease stage.

In summary, the logistic regression analysis demonstrated that disease stages (Stage II, Stage III, and Stage IV) had significant relationships with miRNA-21 changes, as

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indicated by the significant effects and positive effect sizes in both the Delta Ct and Relative Fold Rise models. The findings suggest that as the disease stage progresses, there is an increase in the odds of miRNA-21 changes, providing important insights into the association between miRNA-21 and disease progression.

Table 5: Logistic Regression Analysis Showing Relationship of miRNA-21 changes with Disease Stages

Dependent Variable		B	SE	t	p-vlaue	effect size	R2
Delta Ct	Intercept	8.96	0.37	24.17	0.000	0.899	0.462
	Stage II	4.79	1.02	4.71	0.000	0.252	
	Stage III	5.76	1.02	5.67	0.000	0.328	
	Stage IV	6.86	1.93	3.56	0.001	0.161	
	Stage I	Ref.					
Relative Fold Rise	Intercept	5.85	2.52	2.32	0.023	0.075	0.723
	Stage II	38.45	6.90	5.57	0.000	0.320	
	Stage III	61.95	6.90	8.97	0.000	0.550	
	Stage IV	122.47	13.10	9.35	0.000	0.570	
	Stage I	Ref.					

Ordinal Regression Analysis for Predicting Disease Stage by miRNA-21

The above model revealed the following cut offs for stage estimation using delta Ctvalues

stage I is predicted if delta Ct ≤ 13.31 (16.83/1.264)

stage II is predicted if 13.31 < delta Ct ≤ 14.66

stage III is predicted if 14.66 < delta Ct ≤ 16.69

stage IV is predicted if delta Ct > 16.69

Further the following cut offs for stage estimation was estimated using Relative Rise (RR) values

stage I is predicted if RR ≤ 41.26 (2.867/0.07)

stage II is predicted if 41.26 < RR ≤ 67.10

stage III is predicted if 67.10 < RR ≤ 123.15

stage IV is predicted if RR > 123.15

The ordinal regression analysis aimed to predict the disease stage based on miRNA-21 levels. The dependent variable was the disease stage, while the independent variables were the miRNA-21 levels measured as DeltaCt and RelativeRise. The analysis involved estimating the thresholds and assessing the significance of the predictors.

For the DeltaCt model, the estimated thresholds for each disease stage were as follows: Stage I (16.826), Stage II (18.534), and Stage III (21.090). These thresholds represent the cutoff points for transitioning between disease stages. The estimated thresholds were statistically significant with p-values less than 0.001. The Nagelkerke R2 value for this model was 0.669, indicating that approximately 66.9% of the variance in the disease stage could be explained by the model. Furthermore, the DeltaCt variable had an estimated coefficient of 1.264, indicating that a one-unit increase in DeltaCt was associated with an increased odds ratio of moving to a higher disease stage.

For the RelativeRise model, the estimated thresholds for each disease stage were as follows: Stage I (2.867), Stage II (4.663), and Stage III (8.559). Similar to the DeltaCt model, these thresholds were statistically significant with p-values less than 0.001. The Nagelkerke R2 value for this model was 0.649, suggesting that approximately 64.9% of the variance in the disease stage could be explained by the model.

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The RelativeRise variable had an estimated coefficient of 0.070, indicating that a one-unit increase in RelativeRise was associated with an increased odds ratio of moving to a higher disease stage.

In summary, the ordinal regression analysis revealed significant relationships between miRNA-21 levels (measured as DeltaCt and RelativeRise) and the predicted disease stage. Both DeltaCt and RelativeRise variables were significant predictors of disease stage, as indicated by their estimated coefficients and significant p-values. The estimated thresholds for each disease stage provided valuable information for identifying transitions between stages. The Nagelkerke R2 values indicated moderate to strong explanatory power of the models, suggesting that the miRNA-21 levels contribute significantly to predicting the disease stage.

Table 6: Ordinal Regression Analysis for Predicting Disease Stage by miRNA-21

Ordinal Regression		Estimate	SE	p-value	Nagelkerke R2
Threshold	Stage I	16.826	3.955	<0.001	0.669
	Stage II	18.534	4.194	<0.001	
	Stage III	21.090	4.418	<0.001	
	Stage IV	Ref			
Location	DeltaCt	1.264	.293	<0.001	
Threshold	Stage I	2.867	.538	<0.001	0.649
	Stage II	4.663	.866	<0.001	
	Stage III	8.559	1.654	<0.001	
	Stage IV	Ref			
Location	RelativeRise	.070	.013	<0.001	

IV. DISCUSSION

Given its significant impact on various aspects of OSCC progression, miR-21 has emerged as a potential diagnostic and prognostic biomarker in OSCC. Additionally, researchers have explored its potential as a therapeutic target, and strategies to inhibit miR-21 activity have been investigated to suppress tumor growth.

Our studyobserved thenotable differences among age groups, with individuals aged 30 to 39 years exhibiting 4 cases of SCC (6.3%) and no instances of adenocarcinoma (0.0%). However, the statistical analysis did not establish a significant association between age and the occurrence of SCC and adenocarcinoma.In terms of sex, males exhibited a higher incidence of SCC (84.4%), with all cases of adenocarcinoma (100.0%) found in males. However, the statistical analysis did not reveal a significant association between sex and the occurrence of SCC and adenocarcinoma.

In the studies conducted by various researchers, the focus was on understanding the differences in oral squamous cell carcinoma (OSCC) based on age groups and sex. Coinciding Findings by Suresh GM et al found a higher incidence of SCC in males compared to females. The studies also noted that all cases of adenocarcinoma were found in males, indicating a potential gender disparity in the occurrence of specific subtypes of oral cancer [12]. Brito RTD et al also observed a higher proportion of men in their study samples. This aligns with the prevailing literature suggesting that male individuals are more susceptible to OSCC, possibly due to factors like smoking and alcohol consumption [13]. Our study did not establish a significant association between age and the occurrence of SCC and adenocarcinoma. This contrasts with the study by Chen S et al, which found a significantly higher percentage of females in the younger age group compared to the older group. However, Chen S et al did not

find a difference in TNM classification or tumor stage between the two age groups [14]. The study by Suresh GM et al reported a statistically significant difference in survival rates between genders, while other reports (Liu et al. and Rogers et al.) did not find a significant difference [15,16]. This indicates conflicting evidence regarding the impact of gender on disease-specific survival rates in OSCC. In summary, there are coinciding findings regarding the higher incidence of SCC in males and a higher proportion of men in the study samples. However, there are contradicting findings regarding the association between age and the occurrence of OSCC, as well as the impact of gender on survival rates. These discrepancies highlight the complex nature of OSCC and the need for further research to better understand the role of age and sex in its development and prognosis.

Regarding the site variable, our study observed that the highest prevalence of SCC and adenocarcinoma was observed in the buccal mucosa, with 32 cases of SCC (50.0%) and 3 cases of adenocarcinoma (50.0%). However, the distribution of cases across different sites did not reach statistical significance.Coinciding results were found by Vergers JW both who observed a higher prevalence of SCC in the buccal mucosa. He highlighted that buccal mucosa cancer primarily occurs along the occlusal plane, characterized by pain and ulceration, with the presence of a buccal mass. This suggests a potential link between SCC occurrence and specific clinical characteristics in the buccal mucosa [17]. The study by Kuk SK et al found that buccal mucosa is the fourth most common site for oral SCC in Koreans, following the mandible, tongue, and maxilla [18]. Additionally, Malik A et al reported an increased occurrence of tongue and buccal mucosa cancer in India, indicating that this site is of significant concern in certain populations [19]. Contradicting Findings by others suggested that the distribution of cases across different sites in our study did not reach statistical significance. This implies that while a higher prevalence of SCC and adenocarcinoma was observed in the buccal mucosa, it may not be significantly different from other sites in terms of overall occurrence. This contrasts with findings from other studies that specifically identify buccal mucosa as a prevalent site for oral cancer. Murugesan A et al [20] also contradicting results that oral cancer is most prevalent at the base of the tongue and the base of the mouth, while Singh V et al identified buccal mucosa, alveolus, and the base of the mouth as the most prevalent sites for the occurrence of oral cancer [21]. Bhola R et al also found buccal mucosa to be the most common presented site (23%), followed by other areas of the oral cavity. These differing observations suggest variations in the prevalence of oral cancer sites across different populations and regions [22]. To gain a comprehensive understanding of oral cancer occurrence, future studies should consider these differences and investigate the underlying factors contributing to site-specific variations. This will aid in tailoring prevention and management strategies based on the specific risk profiles of different populations.

Our analysis also indicated a slight increase in mean Delta Ct values with age, but no significant association was found. Similarly, sex and group did not show significant associations with miRNA-21 levels.The logistic regression analysis demonstrated that disease stages (Stage II, Stage III, and Stage IV) had significant relationships with miRNA-21 changes, as indicated by significant effects and positive effect sizes in both the Delta Ct and Relative Fold Rise models which suggested that as the disease stage progresses, there is an increase in the odds of miRNA-21 changes, providing important insights into the association between miRNA-21 and disease progression. The ordinal regression analysis aimed to predict the disease stage based on miRNA-21 levels. Both Delta Ct and Relative Rise variables were significant predictors of disease stage, with estimated coefficients and significant p-values. The estimated thresholds for each disease stage provided valuable information for identifying transitions between stages.The study by Uma

Maheswari TN found coinciding results with statistically significant differences in the mean fold difference of miRNA-21 values among participants with no dysplasia and controls and among participants with severe dysplasia and controls. This suggests that miRNA-21 may have a role in dysplasia progression and could potentially serve as a biomarker for disease severity [23]. Contradicting study found no significant association between age, sex, and group with miRNA-21 levels. She also found no statistically significant difference in the mean fold difference of miRNA-21 between participants with mild and moderate dysplasia and controls. This contrasts with the significant differences observed in other dysplasia groups. Again, differences in the study population and methodology may explain these discrepancies. Similarly, Li J et al also found miRNA-21 to be highly expressed in advanced tongue squamous cell carcinomas compared to early-stage tumors. This observation suggests a potential association between miRNA-21 expression and tumor progression in oral cancer, but it may not directly correlate with other conditions or diseases [24].

In conclusion, the study provides valuable insights into the distribution of SCC and Adenocarcinoma cases across different demographic and clinical variables. Additionally, it highlights the potential association between miRNA-21 levels and disease progression, indicating the importance of miRNA-21 as a potential biomarker for further research and clinical considerations in oral cancer. However, further studies with larger sample sizes are needed to validate and generalize these findings.

V. CONCLUSION

The findings of this study provide valuable insights into the association between miRNA-21 expression and oral cancer, especially SCC and Adenocarcinoma. The results highlight the potential of miRNA-21 as a biomarker for disease progression and severity, which could aid in early diagnosis and prognosis of oral cancer. However, further research is warranted to validate these findings and explore the functional implications of miRNA-21 in oral cancer development and progression. Understanding the molecular mechanisms and biological pathways involving miRNA-21 may open new avenues for targeted therapies and personalized treatment approaches in oral cancer management.

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