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EVALUATION OF EXPRESSION OF MIR-21 AND MIR-548 IN ORAL SQUAMOUS CELL CARCINOMA (OSCC)

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Abstract

Background: microRNAs (miRNAs) are a group of short non-coding RNAs with 18-22 bp length, this category of genes plays critical roles in cellular pathways and dysregulation of their expression is related to the development and progression of different types of cancer including OSCC. This study evaluated miR-21 and miR-548 expression patterns in OSCC samples and these genes' potential as diagnostic biomarkers for oral squamous cell carcinoma (OSCC).

Method: In this cross-sectional study, tumor and marginal tissues were collected from 100 patients with OSCC. Following RNA extraction, miR-21 and miR-548 expression levels were measured using SYBR green master mix and real-time quantitative PCR.

Results: Our study revealed a notable rise in miR-21 and miR-548 expression levels in tumor tissue samples compared to marginal tissue samples. Additionally, miRNA expression was linked to specific clinicopathological features of the patients. ROC curve analysis indicates that only the expression of miR-21 can be used as a biomarker for OSCC.

Conclusions: Our data showed upregulation of miR-21 and miR-548. However, based on our findings, only miR-21 expression could serve as a promising diagnostic and prognostic biomarker in OSCC. Further research is necessary to validate this assertion.

Keywords*:* miR-21, miR-548, Gene expression, oral squamous cell carcinoma, biomarker, micro-RNA

Introduction

Oral squamous cell carcinoma (OSCC) is the primary form of oral malignancy, ranked as the eighth most widespread cancer globally. It is more prevalent in low-income communities, with men over 45 years old being the primary demographic affected. OSCC commonly affects the lips and tongue in clinical cases. (1-2). DNA mutations in the oral keratinocyte are the main source of OSCC, where DNA mutations can be spontaneous, but the presence of mutagens can increase the mutation rate. Plus, to mutations different genetic variations, including single nucleotide polymorphisms (SNPs), may also affect risk in genes that act as a part of pathways involved in carcinogen metabolism, DNA repair, and other protective mechanisms, which may help explain differential susceptibility to OSCC (3-4). Generally, lifestyle factors such as tobacco and alcohol consumption, sunlight exposure, ionizing radiation, and certain viruses like human papillomavirus (HPV) and human immunodeficiency virus (HIV) are considered the primary risk factors for OSCC. These factors primarily contribute to elevated mutation rates in keratinocytes (5-6). Despite focusing on molecular

studies to reveal the mechanism of OSCC progression the fundamental molecular mechanisms of OSCC tumorigenesis are not fully clarified yet. Consequently, additional investigation of molecular changes in OSCC development is crucial to finding novel and efficient ways for early diagnosis and treatment of Oral squamous cell carcinoma (7-8).

Genomic sequencing projects have revealed that many genome positions produce non-coding RNAs. Micro RNAs (miRNAs) are short non-coding RNAs, typically 18-22 nucleotides long, responsible for regulating human gene expression. Notably, a single miRNA can influence the expression of multiple target mRNAs, while a single mRNA can be targeted by several miRNAs. The complementarity between miRNA binding sites and mRNA sequences plays a crucial role in determining the outcome of their interaction. Disruption in the balance of miRNA expression can have severe consequences, such as contributing to disorders like cancer. Numerous studies have elucidated their involvement in cancer-related processes like metastasis, apoptosis, invasion, and EMT (11-12). There are two classes of miRNAs depending on their roles in the cancer cell's progressions, suppressor miRNAs which reduce the expression levels of oncogenes, and oncogenic miRNAs which inhibit the expression of tumor suppressor genes. The consequence of miRNA activity depends on the activity of their target genes (13).

Functional studies in cancer cell lines indicate that the upregulation of miR-21 significantly contributes to cancer advancement. Past research conducted on different cancer cell lines and animal models has demonstrated that elevated miR-21 expression promotes tumor development and invasion while inhibiting apoptosis, hence placing this gene in the oncomiRs classification. A variety of genes are classified as miR-21 target genes, among them PTEN, PDCD4, and BTG2, which hold special significance in cancer cell biology (14). In spite of miR-21 results about the roles of miR-548 in cancer are inconsistent. For example, in a study on breast cancer, inducing miR-548 was discovered to reduce cancer cell migration and viability by targeting the ECHS1 gene. Conversely, in gastric cancer, upregulating miR-548 was found to suppress the RSK-4 gene expression, enhancing cancer cell migration. (15-16).

Given the significance of micro-RNAs in different molecular mechanisms that contribute to tumorigenesis and the existing gaps in our understanding of oral squamous cell carcinoma biology, this research seeks to explore the imbalances of miR-21 and miR-548 in OSCC. The focus is on determining their diagnostic utility to enhance insights into OSCC pathogenesis for prompt and efficient diagnosis.

Material and Method

Study subject:

This study involved 100 patients diagnosed with oral squamous cell carcinoma (68 men, 32 women) of Turkish ethnicity in Iran, with a mean age of 61 ± 4.47 years. All participants were histologically confirmed with OSCC and treated at Imam Reza Hospital, Tabriz University of Medical Sciences, between 2019 and 2023. Tumor and control tissues were collected during surgery for RNA extraction. Exclusions were made for those with prior radiotherapy, chemotherapy, or who declined to participate. Clinicopathological data are summarized in Table 1, and the study was approved by the Local Ethical Committee of ……., and written informed consent was obtained through all subjects.

Extraction of total RNA

Total RNA isolation from tumoral and marginal tissues was carried out with the AllPrep DNA/RNA/Protein kit (Qiagen, Hilden, Germany) per the manufacturer's guidelines. RNA concentration and purity were assessed using a UV spectrophotometer (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA) at 260/280 nm. Subsequently, RNA samples were frozen at -80°C for cDNA synthesis completion.

Synthesizing cDNA and conducting real-time PCR

Real-time polymerase chain reaction (PCR) was conducted for quantitative measuring the microRNAs expression level. In the first step, cDNA was synthesized using total RNA and miRNA-specific primer according to the Chen et al method (17) using the BioFACT cDNA synthesis kit

(BioFACT, Korea). In the next step quantitative real-time PCR was accomplished by BioFACT SYBR Green Master Mix (BioFACT, Korea) in addition U6 gene was used as internal control for normalizing transcript levels of target genes. Primer sequences are presented in Table 2.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA). Differences in expression levels between OSCC tissues and their paired marginal tissues were assessed for statistical significance using Student's t-test. The normality of the data was evaluated using Kolmogorov-Smirnov's normality test. Paired Student's t-test, independent sample t-test, or ANOVA test was used to compare the groups in internal samples. The relationship between the expression of target genes and the patient's clinical parameters was assessed using Pearson's correlation test. All results were reported as mean ± standard deviation (SD). The statistical significance level for all P values was less than 0.05. Receiver operating characteristic (ROC) curve analysis was conducted to investigate the potential of target gene expression as a diagnostic biomarker by assessing its ability to distinguish between groups. Expression values for OSCC tumor samples and normal marginal tissue samples were considered as patient and control values. ROC curve analysis was performed using GraphPad 6 Prism software to evaluate the area under the curve (AUC) at a confidence interval (CI) of 95%.

Results

In this study, we assessed the expression of miR-21 and miR-548 in 100 OSCC patients' samples and corresponding noncancerous tissues. We also examined how the expression of these miRNAs correlated with various clinicopathological parameters in OSCC patients, including gender, age, smoking status, tumor stage, tumor location, differentiation, and distant metastasis. Our findings demonstrated a significant up-regulation of miR-21 expression in tumor samples compared to non-tumor marginal samples (fold change = 1.31, p-value = 0.0005) (Figure 1). Additionally, high miR-21 expression levels were linked significantly to lymph node metastasis (p-value = 0.005), differentiation (p-value = 0.0045), and distant metastases (p-value = 0.0081), but no significant associations were observed with gender, age, smoking, or tumor location. Regarding miR-548 expression, similar to miR-21, this gene showed a significant up-regulation in tumor samples compared to border samples (fold change = 1.12, p-value = 0.015) (Figure 1). Furthermore, miR-548 expression was notably associated with lymph node metastasis (p-value = 0.042), with no significant correlations found with gender, age, smoking, metastasis, differentiation, or tumor location. Detailed data are presented in Table 1.

The Capability of miR -21 and miR-548 for the Diagnosis of OSCC

The ROC curve assessed miR-21 and miR-548's sensitivity and specificity as potential OSCC biomarkers. AROC for miR-21 in OSCC patients was 0.69 and for miR-548, 0.59. See Fig. 2 for ROC curve details.

Discussion

In our study, we observed an increase in miR-21 and miR-548 levels in OSCC tumor tissues compared to normal marginal samples. We also noted that miR-21 expression correlates with differentiation, metastasis, and lymph node invasion, while miR-548 is specifically linked to lymph node invasion. Our findings indicate that both miR-548 and miR-21 could have prognostic implications. Additionally, our ROC curve analysis revealed that dysregulation of miR-21 alone could potentially serve as a diagnostic biomarker for OSCC. This suggests that miR-21 expression levels effectively distinguish OSCC from normal tissue. These results underscore the diagnostic significance of microRNAs in detecting OSCC and emphasize their potential as biomarkers (1). Oral squamous cell carcinoma, the eighth most common cancer globally, remains a significant cause of morbidity and mortality in head and neck cancer patients. Factors such as tobacco use, smoking, alcohol consumption, and betel quid increase the risk of developing OSCC. This type of cancer predominantly impacts men over women and has a higher incidence in the mandible compared to the maxilla, with

a ratio of 3:2. In 2020, there were 377,713 reported cases of OSCC worldwide. The Global Cancer Observatory (GCO) predicts a 40% increase in the frequency of OSCC by 2040, leading to a rise in mortality rates. OSCC is characterized by genetic mutations in keratinocytes causing damage to oral epithelial cells at a molecular level.

Several studies have shown that miRNAs can serve as both diagnostic biomarkers and therapeutic tools in cancer. The impact of miRNAs on gene expression plays a crucial role in delineating diverse clinical characteristics of cancer types. As a result, miRNAs hold promise in the advancement of tumor biomarkers for prognostic forecasts and therapeutic interventions across various malignancies. Given the significant function of micro RNAs in cancer progression and their genetic robustness, they are being considered as potential candidates for early malignancy detection.

This research specifically examines miR-21 and miR-548 as micro RNAs previously linked to cancer. Nonetheless, the involvement of miR-548-3p and miR-21 in OSCC development remains unreported (14-16). miR-21 is widely known as an oncogene in various cancers, with its up-regulation linked to tumor features like metastasis, poor prognosis, and accelerated growth and proliferation of cancer cells. In lung cancer, analysis shows elevated miR-21 gene levels in tumor tissues, correlating with clinical factors such as metastasis and lymph node involvement (20-22). Similarly, research on gastric cancer indicates increased miR-21 expression in malignant tissues, associated with patient characteristics like metastasis and TNM staging, potentially serving as a prognostic indicator. Studies also reveal that inhibiting miR-21 is linked to enhanced apoptosis, reduced metastasis and migration rates, and improved response to chemotherapy (23).

miR-548, a primate-specific miRNA gene family, plays a crucial role in various biological processes. Within this family, miR-548-3p is implicated in cancer pathogenesis. Shi et al. reported a significant decrease in miR-548a-3p levels in breast cancer, linking it to ECHS1 gene regulation, thus hindering tumor cell proliferation and promoting apoptosis. Wang et al.'s research on NSLC cell lines demonstrated that miR-548a-3p overexpression in lung cancer cells elevates apoptosis rate and suppresses cell growth. Conversely, Ni et al. identified miR-548-3p as an oncogene in esophageal squamous cell carcinoma (ESCC), where heightened levels inhibit NRIP1 expression, linked to increased migration and invasion of ESCC cells (15,24).

In the present study, we evaluated the expression pattern and diagnostic potential of miR-21 and miR-548 expression for OSCC. Our findings from ROC curve analysis showed that single upregulation of both micro-RNAs examined, but only miR-21 expression, showed promising diagnostic ability in differentiating OSCC tumors from normal samples. According to our findings, the expression levels of miR-21 may serve as novel diagnostic biomarkers for OSCC, enabling earlier differentiation of tumor tissue from normal tissues and better management of this malignancy. However, further studies are needed to validate and confirm our results.

It is crucial to recognize the limitations of the current study while conducting well-designed experiments to achieve strong results. We did not plan confirmatory mechanistic analyses to validate their role in driving tumor behaviors in OSCC cell lines. These limitations underscore the need for additional research to improve our understanding of the molecular mechanisms leading to miR-21 and miR-548 regulation in OSCC.

Conclusion

The results of this study confirm existing data about the molecular characteristics of OSCC. Our study displayed the upregulation of miR-21 and miR-548 in tissues with OSCC.

In addition, miR-21 and miR-548 expressions were found to be associated with some pathological findings in patients. Moreover, due to the ROC curve analysis results miR-21 can be a promising diagnostic marker for OSCC. However, further validation studies are required to explain the exact roles of these micro-RNAs in OSCC progression.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

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Conflict of Interest

None declared.

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Table 1. Sequence of primers used for real time PCR

Gene	**Primer sequence**	**Annealing temp (°C)**
miR-21 primer	F: 5′- CGTGCTTAGCTTATCAGA -3′ R: 5′- CCAGTGCAGGGTCCGAGGTA -3′	60
miR-21 stem loop	5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGATCAACA-3′	-
miR-548-3p	F: 5′- GCCCTCCTGGGACAAAAACCA -3′ R: 5′- CCAGTGCAGGGTCCGAGGTA -3′	60
miR-548-3p stem loop	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC ACTGGATGCAAAAG -3′	-
U6 primer	F: 5′- GCTTCGGCAGCACATATACTAAAAT-3′ R: 5′- CGCTTCACGAATTTGCGTGTCAT -3	60
U6 stem loop	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC ACTGGATACGACAAAAATAT-3′	-

Table 2. Association of transcript levels of miR-21 and miR-548 and clinicopathological features of patients.

Cilinicopathological Character	**Number**		**P value miR-21**	**P value miR-548-3p**
Age	>60	76	0.21	0.32
	<60	24		
Sex	Female	32	0.29	0.38
	Male	68		
Family Story	Positive	9	0.097	0.19
	Negative	91		
Smoking	Positive	62	0.086	0.093
	Negative	38		
Lymph node Invasion	Positive	57	0.005	0.042
	Negative	43		
Distant Metastasis	Positive	8	0.0081	0.067
	Negative	92		
Differentiation	Poor	39	0.0045	0.059

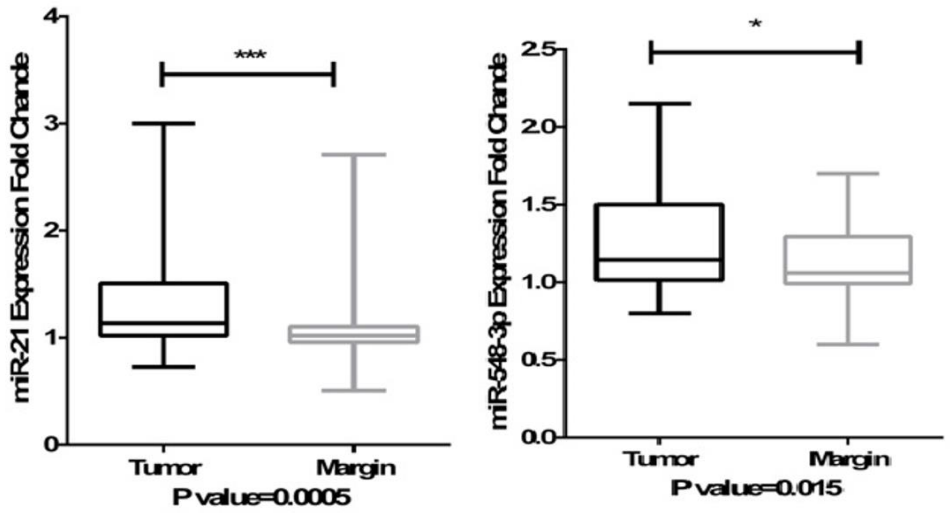

Figure 1. Expression of miR-21 and miR-548 in OSCC

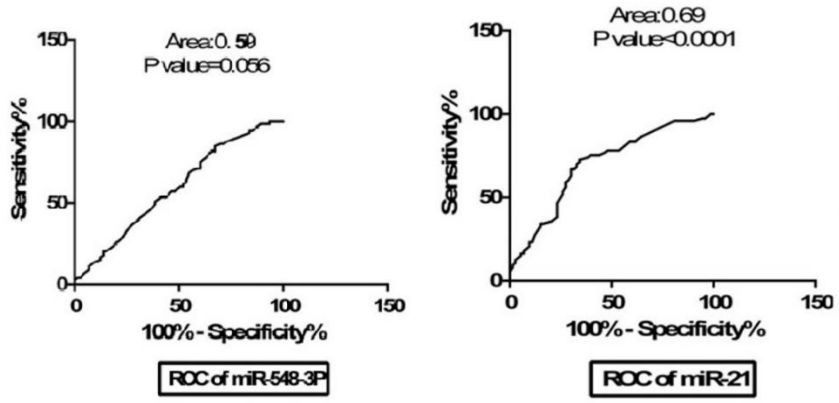

Figure 2. ROC curve analysis of miR-21 and miR-548 expression in OSCC.