miRNA-3651: a Diagnostic Marker in Oral Squamous Cell Carcinoma?

Farzane Saeidmohamadi

Islamic Azad University

Maedeh ghorbanpour

Islamic Azad University

Maryam Jolehar (✉ joleharm@gmail.com)

Islamic Azad University of Tehran: Islamic Azad University Central Tehran Branch

<https://orcid.org/0000-0001-8873-4401>

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Abstract

Background and aim: microRNAs are a group of small non-coding single-stranded RNAs that control post-transcriptional gene expression. They can act as oncogenes or tumor suppressors by amplifying or preventing the expression of certain genes. present study was conducted to assess the expression of miRNA-3651 in paraffin blocks of oral squamous cell carcinoma (OSCC) cells using qRT-PCR method in Islamic Azad university, dental branch of Tehran in 1399.

Material and methods: This case-control study was conducted on 20 paraffin blocks of irritation fibroma (IF) as control group and 20 paraffin blocks of patients with oral squamous cell carcinoma as case group. After RNA extraction, qRT-PCR was performed. all experiments were repeated 3 times for each sample. Eventually the data were analyzed by SPSS 24 statistics software. Differences in miRNA expression levels between the two groups were compared using the independent samples t-test. In order to evaluate the correlation between mean levels of miRNA-3651 marker and different variables (grade, age and sex of patients), Kruskal-Wallis test, Pearson's correlation coefficient test and independent samples t-test were used, respectively.

Results: The results showed that the mean expression of this biomarker method was 10.15 ± 5.44 rpm in normal tissue and 8.11 ± 1.57 rpm in cancerous tissue. Despite the lower expression of miRNA-3651 in cancerous tissue samples than normal samples, this decrease was not statistically significant ($P > 0.05$). There was no significant difference between the mean level of miRNA-3651 marker and different grades, age and sex of patients ($p > 0.05$).

Conclusion: In the end, it seems that the evaluation of changes in the expression of miRNAs such as miRNA-3651, can be a minimally invasive method, in early detection and screening of patients with OSCC. Decreased expression of miRNA-3651 marker in cancerous tissue compared to normal tissue indicates the importance of these biomarkers, including miRNA-3651 in the diagnosis of oral cancer, and the researchers of this project suggest further and broader investigations on the mechanism of action and signaling cascades associated with this marker in oral cancer.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors of the head and neck and accounts for more than 90% of oral malignancies (1). Because 5-year survival rate is directly related to disease staging at diagnosis, preventive measures and early intervention treatments reduce or even stop the progression of the disease (2, 3). Changes in the occurrence of biomarkers can be helpful in diagnosis, prognosis, and treatment. However, the lack of clinically proven biomarkers still limits treatment decisions. Because most biomarkers lack the necessary specificity and sensitivity and cannot be used as reliable tools (4, 5).

MicroRNAs (miRNAs) are a group of small non-coding single-stranded RNAs with a length of about 20 to 22 nucleotides that regulate gene expression after transcription (6). They are responsible for controlling

the expression of 50% of all genes (7) and are vital regulators of cellular processes such as proliferation, apoptosis, cell differentiation and movement (8). Depending on the type of tumor, they can act as oncogenes or anti-oncogenes by amplifying or preventing the expression of certain genes and suppressing the host's immune response to cancer cells, which is one of the most important steps in tumorigenesis (9). MiRNAs are known for their role in cell growth and proliferation and regulation of vital pathways for cancer development (10). MiRNAs are thought to be potential biomarkers for various solid tumors, including OSCC (11, 12). As a result, they can be used as useful minimally invasive tools for early diagnosis, screening, follow-up of therapeutic responses, and diagnosis of relapse (13, 14). MiRNAs are secreted from cells and enter the bloodstream. They are found in various body fluids including plasma, serum, blood cells and saliva (15). Therefore, identifying altered patterns of miRNAs in blood and tumor tissue can be considered as biomarkers of cancer (24–26). Changes in the expression of miRNA-3651 in a number of cancers have been studied (25, 26). So far, very few studies (only 3 cases) have been performed on the expression of miRNA-3651 in oral cancers (26–28). Due to the lack of information in this field and the importance of early diagnosis of OSCC for treatment outcome, the present study aimed to evaluate the expression of miRNA-3651 on paraffin blocks of oral squamous cell carcinoma in comparison with normal mucosa in the Department of Oral and Maxillofacial Pathology of Dentistry Faculty of Islamic Azad University of Tehran in 1399.

Materials And Methods

20 paraffin blocks fixed in formalin (FFPE) for patients with oral squamous cell carcinoma from the archive of Dental Research Center, Dentistry Research Institute of TUMS as case group and 20 paraffin blocks of irritation fibroma (IF) from the Department of Oral and Maxillofacial Pathology of the Dentistry Faculty of the Islamic Azad University of Tehran was prepared and collected as control group. The expression of miRNA-3651 was determined by qRT-PCR method in the mentioned tissues.

conducting research:

RNA isolation stage

Total-RNA was extracted from tissues using RiboEX (genall south korea) RNA extraction kit based on the manufacturer's protocol. RNA was determined using the A260 / A280 adsorption ratio using a Thermo scientific-Nanodrop 2000 spectrophotometer.

QRT-PCR

Reverse transcription was performed using the all-in-one miRNA qRT-PCR diagnostic kit. The qRT-PCR step was performed using the qRT-PCR mRNA detection kit. The RT reaction system consisted of 1µl of 2.5 U / µl poly A polymerase, 1ul of RTase mixture and 5µl of PAP / RT × 5 buffer, and 2µg total RNA template and ddH2O without RNase / DNase. The reaction was performed at 37 ° C for 60 minutes and at

85 ° C for 5 minutes. 20 ul reaction system for PCR containing 10µl of qPCR × 2 mixture, 2µl of First-strand cDNA (1: 5 dilution), 2 µl of universal adapter PCR primer and 2ul of all-in-one miRNA qPCR primer and 4 µl of Was ddH2O. Amplification of the reaction was performed under the following conditions: 95 ° C for 10 minutes and 40 cycles of 95 ° C for 10 seconds, 60 ° C for 20 seconds and 72 ° C for 10 seconds. A monochrome BT-PCR RT-PCR system was used for amplification. MiRNA expression levels were normalized to U6 small nuclear RNA. Alteration of miRNA expression levels was performed by 2^{-ΔCt} method in paraffin blocks. For each sample, all experiments were repeated 3 times (25).

Statistical reviews:

Data were entered into SPSS 24 statistical software. Normality test was performed by Kolmogorov-Smirnov test. Since the distribution of ΔCT values was not significantly different from the normal distribution, t-test Independent samples were used to compare the mean miRNA expression in IF and OSCC samples. The obtained P-value indicated the significance of the study. The correlation between miRNA and histopathological grade of cancer cells was evaluated by Kruskal-Wallis test. The relationship between patients' age and miRNA-3651 marker was evaluated using Pearson correlation coefficient test. Also, the relationship between patients' sex and miRNA-3651 marker was examined using Independent samples t-test.

Results

The mean age of the patients was 69.05±14.76. The minimum age of the patients is 33 years and the maximum age is 92 years.

In this study, 40% of patients were female and 60% were men.

Table 1
Descriptive evaluation of tumor size
(T)

Tumor size	count	percentage
T1	1	5
T2,NOS	5	25
T3	5	25
T4.NOS	1	5
T4a	7	35
T4b	1	5
Total	20	100

T1 ≤ 2cm in diameter, T2 ≥ 2cm & < 4cm, T3 > 4cm, T4a= Moderately advanced local disease, T4b= Very advanced local disease, X= cannot be measured, NOS= not otherwise specified

Table 2
Descriptive study of involvement around lymph nodes around tumor (N)

Lymph node involvement	count	percentage
Nx	8	40
N0	8	40
N1.NOS	1	5
N2a	1	5
N2b	2	10
Total	20	100

Nx= Regional lymph nodes cannot be assessed, N0= No regional lymph node metastasis, N1= Metastasis in a single ipsilateral node ≤3 cm in dimension, N2a= Metastasis in a single ipsilateral node ≥3 cm & ≤ 6 cm in dimension, N2b= Metastasis in multiple ipsilateral nodes, none > 6 cm in dimension

Table 3
Descriptive study of metastasis to tissues around tumor (M)

metastasis	count	percentage
Mx	7	35
M0	13	65
Total	20	100

Mx: Metastasis cannot be measured, M0= No distant metastasis, M1= Distant metastasis

Table 4
Evaluation of grade of cancerous tissues

grading	count	percentage
Grade I (Well differentiated)	10	50
Grade II (Moderately differentiated)	7	35
Grade III (Poorly differentiated)	1	5
Grade X (Unknown)	2	10
Total	20	100

Using The Kolmogorov-Smirnov test, we investigated the significance of ΔCT scores in each of the cancerous and normal tissues. This test showed that the distribution of ΔCT values was not significantly different from normal distribution ($p > 0.05$) and therefore, normal distribution for these values can be accepted in each group. This result can also be seen from the graph below. This result shows that the use of independent samples parametric t-test to investigate the mean difference of ΔCT between the two groups is acceptable.

Table 5
Evaluation of Significance of ΔCT difference
between cancerous and normal tissue
groups

average		10.15
normal	Test statistic value	0.16
	p-value	0.18
	average	8.11
cancerous	Test statistic value	0.08
	p-value	0.20

The results of this test showed that there is no significant difference between the mean ΔCT score between normal and cancerous tissue groups ($p = 0.12$). Results are shown in the table and diagram below.

Table 6
Comparison of mean difference of ΔCT (expression level of miRNA 3651) between the
two groups

tissue	count	mean	standard deviation	T statistic value	p-value
IF	20	10.1518	5.4400	1.60	0.12
Cancerous	20	8.1188	1.5769		

To evaluate the predictive accuracy of the miRNA-3651 marker in the diagnosis of oral cancer, the ROC diagram (receiver operating characteristic curve) and the area under the curve (AUC) were used. Youden index is also used to access the best cut-off point. The ROC curve is shown below.

Table 7
Youden index value

Estimation of the area under the curve (AUC)	0/34
Deviation from the mean	0/10
p-value	0/099
Youden index value	0/30

The area under the curve is 0.34 which is not significantly different from the number 0.50 ($p > 0.05$). This result indicates that the miRNA-3651 marker does not have high predictive accuracy in the diagnosis of oral cancer. This result is consistent with what was obtained from the independent samples t-test in the previous section. Also, the value of the Youden index for a shear point with a sensitivity of 100% and a specificity of 30% is equal to 30%, which is not an acceptable value for proper predictive power of a diagnostic tool.

According to the result of Kruskal-Wallis test, there was no significant difference between the mean level of miRNA-3651 marker and different histopathologic grades ($p = 0.77$). According to the result of Pearson's correlation coefficient test, the relationship between patients' age and mean level of miRNA-3651 was not significant ($p > 0.05$). According to the results of independent samples t-test, the difference between the mean level of miRNA-3651 marker between male and female patients was not significant ($p = 0.18$).

Discussion

According to the human genome, miRNA-3651 is a non-coding RNA gene found in various classes of eukaryotes, including animals. This miRNA is involved in regulating gene expression after transcription. It is located in the nucleus of a human cell on chromosome 9 (9q22.31) and is estimated to be 24 nucleotides in length. Changes in the expression of this miRNA have been studied in various cancers, including esophageal cancer (25), nasopharynx (29), liver cells (32) and colorectal (30, 33). Also, according to articles that examined such biomarkers in precancerous lesions compared to cancerous and normal tissue, and observed changes in the incidence of these biomarkers (31), resulting in the possibility of changes in the expression of this miRNA in cancer. The squamous cells of the mouth also made sense. Therefore, the aim of this study was to investigate the expression of miRNA-3651 on paraffin blocks of squamous cell carcinoma of healthy mouth and mucosa by qRT-PCR method. The results of this study showed that the mean incidence of this biomarker according to the computational method ΔCT in normal tissue was 10.15 ± 5.44 rpm and in cancerous tissue was 8.11 ± 1.57 rpm. Despite the lower expression of miRNA-3651 in cancer tissue samples than normal samples, but this decrease and difference was not statistically significant ($P = 0.12$). Also, the relationship between the expression of this marker and the variables of age, sex and grade was investigated. According to the result of Kruskal-Wallis test, there was no significant difference between the mean level of miRNA-3651 marker and different

grades ($p=0.77$). According to the results of Pearson correlation coefficient test, the relationship between patients' age and miRNA-3651 marker was not significant ($p>0.05$). According to the results of t-test of two independent samples, the difference between the mean level of miRNA-3651 marker between male and female patients was not significant ($p=0.18$).

In a study by Cong Wang et al., The results of measuring the expression levels of miRNA-3651 by qRT-PCR in esophageal squamous cell carcinoma (ESCC) and comparing it with adjacent healthy tissue, as well as examining the relationship between miRNA-3651 expression and clinical signs, showed that The expression of miRNA-3651 in tumor tissues (0.48 ± 0.45) was reduced compared to healthy tissues adjacent to the tumor (1.05 ± 0.07) ($P < 0.001$). It was also shown that the expression of this marker decreases with increasing T stage in patients. Despite the similarities between the two studies and the decrease in the incidence of miRNA-3651 biomarker in cancer cells compared to normal tissue, but this decrease was not statistically significant in the present study. Despite the same laboratory methods (qRT-PCR) in both studies, it should be noted that the cancers studied in these two studies are different, which can be partially different due to the type of mutations and different mechanisms in tumor production. Justified. (25)

In a study by Jutta Ries et al., Changes in the incidence of miRNA-3651, miRNA-494, and miRNA-186 in both tissue and blood samples of patients with OSCC were compared with those of healthy individuals. The study method was similar to the present study, qRT-PCR method, which is one of the common methods in the study of miRNA and has been introduced as the standard Golden Genomic study in many diseases (34). The results of this study did not show a statistically significant difference in the incidence of miRNA-494 and miRNA-186 between the case and control groups. While the expression of biomarker was significantly reduced in tumor tissue compared to mucosa of normal individuals ($p = 0.0001$). The result was consistent with the result of the present study. However, unlike tumor tissue, the expression of the miRNA-3651 biomarker was increased in the blood of cancer patients. Differences in the incidence of biomarkers in the tissues and blood of cancer patients may indicate different functions of miRNA-3651 in the bloodstream and tissues. Finally, this study concluded that reducing the expression of miRNA-3651 in tumor tissues could be a diagnostic biomarker (27).

Another study by Jutta Ries et al., Which examined the incidence of miRNA-3651, miRNA-494, and miRNA-186 only in whole blood samples from people with OSCC compared to healthy volunteers, also found an increase in miRNA-3651 levels. And it was repeated. However, this discrepancy between this study and the present study can be explained by testing and marking this marker on tissue and blood. There was also a significant correlation between miRNA-3651 levels and lymph node status, clinical stage and tumor grade. Given these cases, it was suggested that altered miRNA-3651 levels in patients' blood samples could be used as a minimally invasive method for screening patients (26).

Another study by Jutta Ries et al. Emphasized the importance of changing the incidence of miRNA-3651, miRNA-494, and miRNA-186, even in the blood of patients previously treated with OSCC, to assess the difference in recurrence in patients. In this study, 4 groups consisting of: 1- with initial diagnosis of OSCC

(case group), 2- healthy individuals (control group), 3- individuals with no recurrence of the disease and 4- individuals with disease recurrence. An important finding of this study was that the level of miRNA-3651 in the blood of patients with recurrence of the disease was significantly increased (Pvalue = 0.001). Considering the importance of early detection of recurrence in increasing the probability of patient survival, it can be concluded that the study of the expression of this biomarker in the prevention and early detection of recurrence of the disease is necessary and helpful (28).

In a 2018 study by Schneider et al., MiRNAs were examined in the tissues and serum of people with OSCC compared with healthy people. Of the 225 miRNAs examined in tissue, the results were almost identical to those of the present study. The expression level of miRNA3651 in healthy tissue was 7.24 ± 0.37 and in tumor tissue was 12.6 ± 5.58 and a significant decrease ($p = 0.0068$) was observed in the expression of this marker in oral cancer compared to healthy tissue. In the present study, a decrease in the expression of this marker was observed, although this decrease was not statistically significant (10).

A 2020 study by Tuncer et al., Which examined changes in the expression profile of miRNAs in the peripheral blood of people with ovarian carcinoma compared to healthy individuals, found conflicting results with the results of the present study. The findings of this study showed an increase in the expression of some miRNAs, including miRNA3651. Perhaps this difference in the results obtained with respect to the use of peripheral blood in this study compared to the use of tissue in the present study, two different cancers, ovarian cancer and oral squamous cell carcinoma and specifically the mechanism and activity of different cell cascades in each Attributed to these cancers. But the important point is the change in the expression of this marker that by examining the mechanism of its effect in different cancers, miRNA3651 can be considered a potentially useful marker for early detection of cancers, especially oral cancer (35).

In a study by Li et al. In 2020 entitled The effect of miRNA3651 on promoting the proliferation of colorectal cancer cells through direct inhibition of T box transcription factor 1. In this study, which examined the expression of miRNA-3651 in colorectal cancer tissues compared to healthy tissue, an increase in the expression of this marker was observed in cancer tissues and this increase in expression was related to the TNM stage. The result was inconsistent with the result obtained in the present study. In this study, it was found that reducing the expression of this marker stops the growth of cancer cells and induces apoptosis. Western blot in this study also showed that decreased expression of this marker leads to inactivation of PI3K / AKT and MAPK / ERK signaling pathways in colorectal cancer cells. Finally, this study suggested that this marker has oncogenic potential in colorectal cancer (33).

In a 2019 study by Zhao et al. Entitled The effect of miRNA3651 on promoting the growth and invasion of hepatocellular carcinoma cells targeting PTEN, qRT-PCR was used to evaluate the expression of this marker as well as methods such as transwell invasion Cell invasion) and Western blot were used to evaluate the status of cell apoptosis. In this study, contrary to the present results, an increase in the expression of this marker was observed compared to normal tissue. This increase in expression significantly led to the progression of proliferation and invasion of the Huh-7 cancer cell line. Conversely,

decreased expression of this marker clearly led to inhibition of growth and invasion of this cell line by stimulation of apoptosis. As a result, this study suggested that miRNA3651 could be considered as a new therapeutic target in hepatocellular carcinoma (32).

Mentioning all these articles indicates that miRNA3651 can be used as a potential diagnostic and therapeutic marker in various cancers, although the type of cancer under study and the different cell cascades that are activated in each cancer lead to various changes in the occurrence of this marker. It turns out that more and more careful study of its effect on any cancer can be a new way to help cancer patients.

Conclusion

Although the expression rate of miRNA-3651 in cancerous tissue samples was lower than normal ones, this decrease and difference were not statistically significant ($P>0.05$). There was no significant difference between the mean expression of miRNA-3651 and different histopathological grades, age and sex of the patients ($p>0.05$). It seems that better identification of miRNAs change patterns in tumoral tissue can be considered as cancer biomarkers and as a minimally invasive tool in early diagnosis and screening of cancer patients. The reduction of miRNA-3651 marker expression in cancerous tissue relative to normal tissue indicates the importance of these biomarkers such as miRNA-3651 in the diagnosis of oral cancer, and the researchers of this project suggest further and broader investigations and investigation of the mechanism of action and signaling cascades associated with this marker in oral cancer

Abbreviations

MiRNA: micro-RNA

RNA: Ribonucleic acid

OSCC: Oral squamous cell carcinoma

qRT-PCR: quantitative Reverse transcription polymerase chain reaction

IF: Irritation fibroma

FFPE: Formalin fixed paraffin embedded

TUMS: Tehran university of medical sciences

RTase: Reverse transcriptase

ESCC: Esophagous squamous cell carcinoma

Declarations

Ethics approval and consent to participate : <https://ethics.research.ac.ir/IR.IAU.DENTAL.REC.1399.275>

Consent for publication : Not applicable

Availability of data and materials : The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions : FSm collected and documented all data regarding the archives. FSm performed the laboratory examination. MJ & MGh interpreted the data and wrote the article. All authors contributed in writing article. All authors read and approved the final manuscript.

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Conflict of Interest Disclosures: The authors have no conflicts of interest.

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Figures

Figure 1

Investigation of the normality of data distribution in two groups

Figure 2

box plot diagram showing non-significant difference in miRNA 3651 expression between two groups

Figure 3

ROC curve and the area under the curve show the accuracy of predicting the miRNA 3651 marker