

Investigation of the expression levels of miR-21, miR-132, miR-29a, miR-204, and miR-138 in patient's plasma with primary OSCCs

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ABSTRACT

This is the eighth most malignant tumor in the world, causing the highest incidence and malignancy rate of all cancers in the mouth and maxillofacial region. In cells, miRNAs regulate development, differentiation, proliferation, and differentiation, and miRNA expression is a better indicator of physiological status than DNA expression. miR-21, miR-132, miR-29a, miR-204, and miR-138 levels were measured in plasma from patients with primary OSCC and healthy controls. A Real Time-PCR technique was used to measure miR-21, miR-132, miR-29a, miR-29a, and miR-204 expression levels in plasma from 38 healthy and 38 people with primary OSCC. A standard distribution test and a CT unit were used to confirm the quantitative data on miRNA expression. Gene expression levels were compared between two groups of patients and healthy groups using a Mann-Whitney test and an unpaired t-test. MiR-21's median CT value was 29.68 in the OSCC group and 32.89 in the healthy group, and miR-29a's median CT value was 37.54 and 36.46 in the OSCC group and healthy group, respectively. Additionally, miR-132's CT values were 37.71 and 36.40, miR-138's CT value was 35.37 and 31.21, and miR-204's CT value was 36.44 and 36.17. The results showed that miR-21 expression levels increased significantly, while miR-29a, miR-132, and miR-138 ($P < 0.05$), and miR-204 expression levels did not differ significantly ($P > 0.05$). As a result of this study, the expression levels of microRNAs may be considered to diagnose OSCC at an early stage. It is essential to diagnose this disease early to improve treatment and patient health outcomes.

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Introduction

There are many types of cancer in the mouth and maxillofacial region, but Oral squamous cell carcinoma (OSCC) is the most common and malignant. OSCC is primarily caused by tobacco smoking, alcohol consumption, and viral infections. Surgery, radiotherapy, chemotherapy, and immunotherapy are all used to treat OSCC in the present day (1-4). As a result of these treatments, surrounding normal tissues are also harmed, and these side effects are linked to higher mortality rates (5,6). Identifying and diagnosing OSCC at an early stage is key to reducing the mortality and morbidity of this disease. OSCC can arise from any epithelial dysplasia in the oral environment that has the potential to become malignant. The disease's long-term asymptomatic nature and similarity to inflammatory lesions make two-thirds of OSCC patients already in advanced stages when diagnosed. Histopathological analysis and clinical examinations are used to diagnose OSCC, but they are hidden diagnoses (4, 6-8). These cancers are currently treated using surgery and radiotherapy, which can negatively affect functions such as swallowing and speak-

ing and people's appearance. OSCC should be diagnosed early to reduce complications from primary treatment. In patients with OSCC, the likelihood of recurrence is 15-33%, making the use of appropriate diagnostic methods essential for predicting this risk. Regarding sensitivity and specificity, biopsy and histopathological examinations are still the most reliable methods of diagnosing this type of cancer (2,3,9). Cell division, differentiation, and cell death are all controlled by different regulatory factors. Tumor formation occurs when these genes and related processes are out of control in cancer. Recently, researchers have identified some of the genes and molecular mechanisms associated with tumorigenesis. The miRNA molecules mediate several physiological processes and play an essential role in the development of cancer (3,10). Cell physiological state is better determined by miRNA expression than mRNA (11). Dysregulation of miR expression is closely related to cancer and occurs in fragile genomic locations, leading to genomic instability. As a biomarker, miRNAs estimate physiological and pathological conditions by regulating hundreds of genes. The stability and resistance to the destruction of miRNA molecules make them suitable

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candidates for biomarkers (12). Human serum or plasma can be examined for microRNAs to diagnose diseases (12,13). Therefore, a cancer microRNA profile can be detected in serum or plasma without invasive procedures (14). Different miRNA signatures have been observed in different population cohorts, perhaps due to differences in lifestyle habits, genetics, and other environmental factors. In addition to being potential biomarkers of prognosis, miRNAs could also serve as indicators of treatment response. Liquid biopsy continues to face technical challenges despite recent advances. Purifying and isolating RNA from body fluids is essential and normalizing with an exogenous control (15). In this study, the expression levels of miR-21, miR-132, miR-29a, miR-204, and miR-138 were measured in plasma from patients with primary OSCC and healthy groups.

Materials and Methods

Patients and sampling

This study consisted of 76 patients, with 38 primary OSCC patients and the remaining 38 healthy individuals. Oral and maxillofacial surgery of Tehran hospitals specializing qualified all patients for primary surgical resections. During the evaluation of the cases, squamous cell carcinoma was confirmed pathologically in every case. Before any surgery, all participants gave their informed consent, and no chemoradiation therapy was given to them before the surgery. The presence of any malignancy in the past or presence of malignancy in the previous can be a risk factor for people with SCC. Activation oral infections, hyperglycemia, Sjogren's metabolic syndrome, inflammation and tendon injuries, CADs (coronary artery disease) and CHDs (congenital heart defects) were among the samples with inflammation and tendon injuries, CADs (coronary artery disease) and CHDs (congenital heart defects). Therefore, samples at the end stage of their disease cannot be operated on because they are in the terminal stage. In the study, new cases of OSCC and OSMF were included as well as healthy controls who were willing to participate in the study. Separate serum from blood samples taken from the patients before any surgical intervention was performed. Centrifuges were used to separate the serum from the blood. Clot activator tubes were used to collect blood samples as part of the blood collection process.

RNA extraction

All materials and tools must be RNase/DNase-free to extract RNA. For this reason, RNase/DNase-free containers and tubes had to be autoclaved for 15 minutes at 15 psi pressure before working with RNA. Each sample was collected with 5 ml of venous blood. A centrifuge at 2300 to 2500 rpm was used to centrifuge blood samples after 60 minutes. Plasma was stored at a temperature of 80 degrees Celsius until further analysis. A solution of RiboEx-LS was applied to plasma to extract RNA (GeneAll, South Korea). Three times (600 to 750 microliters) of triazole liquid were added to 200 to 250 microliters of plasma during extraction with RiboEx solution. Incubation at room temperature (15-30°C) for five minutes was performed on the contents of the microtube using a micropipette and a mixing instrument. For every 750 microliters of triazole, add 200 microliters of chloroform. After 15 seconds of vigorous shaking, homogenize the mixture. Centrifuge

the microtube at 12,000 rpm for 25 minutes at 2-8°C, then incubate it at 15-30°C for 2-3 minutes. The centrifugation process produces three phases: The colorless upper phase contains RNA, the middle phase contains protein, and the lower phase contains DNA. The upper phase was slowly transferred into a new tube without an RNase enzyme to prevent contamination of the upper phase with protein and DNA. Incubate at 15-30 degrees Celsius for 20 minutes after adding isopropanol to an amount of liquid that has been transferred (about 500 microliters). Twenty minutes of centrifugation were performed at 2-8 degrees Celsius at 12000 rpm. The upper phase should be drained, ethanol (75% ethanol is prepared with DEPC-treated water), and shaken for 20 seconds. 15-minute centrifugation at 2-8 degrees Celsius at 7500 rpm was performed on the sample at 7500 rpm. The supernatant solution should be drained, the tube should be inverted on a paper towel, and the sediment should be partly dried at room temperature for a few minutes. This technique dissolves the sediment in 40 microliters of DEPC-treated water for 10 minutes at 56 degrees Celsius. RNA samples were transported and stored in a freezer at -80 degrees Celsius. MiRNAs were quantified with the NanoDrop 1000 (NanoDrop, Wilmington, DE).

cDNA synthesis

The Pars Genome kit manufactured in Iran is used in this study to make cDNA from cells. As part of the protocol, we added the following materials to every nuclease-free microtube: First, we normalized the miRNA221 concentration by bringing it to 1000ng/uL after reading the miRNA concentration value from the Nanodrop device. To unwind the nucleotide strands, we incubated the microtube at 65°C for 5 minutes with random hexamer and 1000 ng of miRNA; then, we immediately placed the microtube on ice. A 10 uL batch of RT Master mix enzyme is added after cooling (to prevent premature enzyme action). In the PCR machine, it is heated to 25 °C for 10 minutes, 42 °C for 60 minutes, and 70 °C for 10 minutes following the kit's temperature program. To ensure the accuracy of the cDNA synthesis kit and assess the quality of miRNAs extracted by primers 5S, RT-PCR was performed after cDNA synthesis from RNA samples. We prepared a Master Mix with water, cDNA, and PCR Amplicon for the number of reactions and an additional sample (for control) to perform multiple reactions simultaneously and reduce the possibility of pipetting errors. To ensure that the Master Mix PCR Amplicon is not contaminated, negative control of water, primers, and Amplicon is added inside the PCR along with the other samples. As soon as the reaction was finished, we took the samples and placed them on 1.5% agarose gel according to the desired band size.

Real-time PCR

As instructed by the manufacturer, the SYBR Green master mix (Pars Genom, Iran) was used for RT-PCR using the Applied Biosystems-Step One machine. A 20 µL PCR reaction included 50ng of cDNA, 2 µL universal primers, 10pmol of each primer, and 10 µL of 2× SYBR Green PCR master mix for each PCR reaction. For activation, 95 °C was used for 15 minutes, 40 cycles were included, followed by processing at 95 °C for 10 seconds. Following the primers being connected at 62 °C for 32 seconds, they

were extended for 34 seconds at 72 °C, forming the melting curve by setting the temperature between 60°C and 95°C. After the reaction is complete, a threshold cycle (Ct) is determined for each sample. Using 5s as the control, the $\Delta\Delta C_t$ method was used to evaluate gene expression differences between samples.

The statistical analysis

In order to perform the statistical analysis, GraphPad-Prism (version 9.4, GraphPad Software) was used. Standard deviations and means were used to categorize categorical data. Mann-Whitney and t-Test tests were used to compare serum expression levels of five miRs. A 0.05 level of statistical significance was used.

Results

Demographic information

The demographic information was a total of 38 samples in each group. In the healthy group, 19 were women, 19 were men, and in the OSCC group, 18 were women, and 20 were men (Figure 1). The average age of the studied subjects in the healthy and sick groups was 54 and 55 years, respectively.

Melt curve and amplification curve evaluations

A calculated median CT value of miR-21 was 29.68 in the OSCC and 32.89 in the healthy groups, and a calculated median CT value of miR-29a was 37.54 and 36.46 in the OSCC and healthy groups, respectively. In the OSCC and healthy groups, miR-132 was calculated to have a median CT value of 37.71 and 36.40, respectively. MiR-138 and miR-204 were calculated as 35.37/31.21 and 36.44/36.17, respectively, in the OSCC and the healthy groups. An unpaired t-test was conducted to analyze the data, and the results showed a significant increase in miR-21 expression and a significant decrease in miR-29a, miR-132, and miR-138 expression levels ($P < 0.05$) Figures 2 and 3 show melt curves and amplification plots of products based on microRNAs and housekeeping genes 5 in real time. However, miR-204 expression levels did not differ significantly ($P > 0.05$).

miRs expression level in OSCC plasma and healthy samples

A Melting peak curve was used to interpret the results of three cDNA vials constructed from Evolution reference genes and markers for each individual. The expression

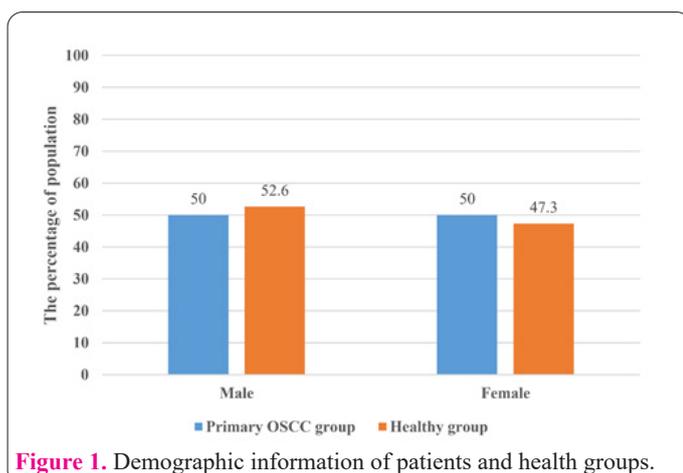


Figure 1. Demographic information of patients and health groups.

of miR-31 was determined using a quantitative real-time PCR assay. There was a significant increase in miR-21 (<0.0001) expression in OSCC samples when compared to healthy controls. As compared to healthy samples, OSCC samples expressed significantly lower levels of miR-29a (<0.0001), miR-132 (<0.0075), miR-138 (<0.0002), and miR-204 (<0.57) (Figure 4). Based on the analysis, there was a significant statistical difference in the expression level of miR-21, miR-29a, miR-132 and miR-138 between the two groups of patients and healthy ($P < 0.05$), whereas the expression of miR-204 did not show any significant differences ($P > 0.05$).

The differences in miRs expression in the OSCC and healthy groups

We calculated the relative differences in expression by using the $\Delta\Delta C_t$ method for miRs. A total of 2.07 times more miR-21 was expressed in OSCC plasma samples than in healthy plasma samples, but miR-29a, miR-132, miR-138, and miR-204 expression in plasma samples from OSCC samples was 8.25 times lower than 3.2 times lower, 5.14 times lower, and 1.16 times lower, respectively (Figure 5).

Discussion

Approximately 9% of cancers present as salivary gland carcinoma, sarcoma, or lymphoma, out of which 92% are squamous cell carcinoma. Advanced stages of oral cancer are most often diagnosed. Due to high progression, these lesions are generally discovered after the emergence of clinical symptoms, resulting in a poor oral cancer prognosis in most countries (17). Human serum or plasma analysis can be used to identify microRNAs as diagnostic

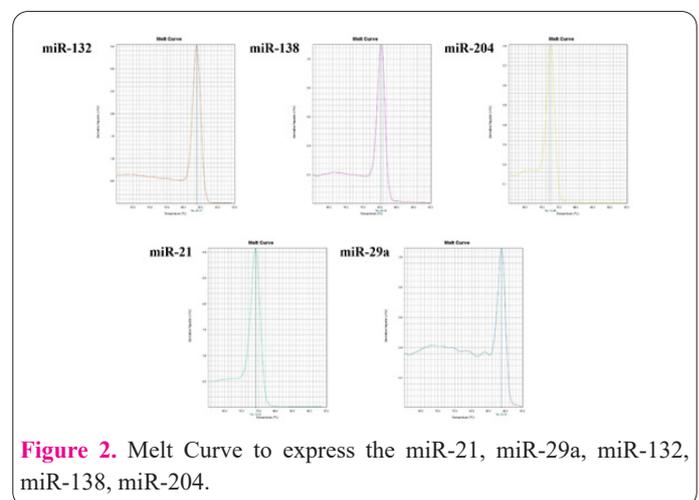


Figure 2. Melt Curve to express the miR-21, miR-29a, miR-132, miR-138, miR-204.

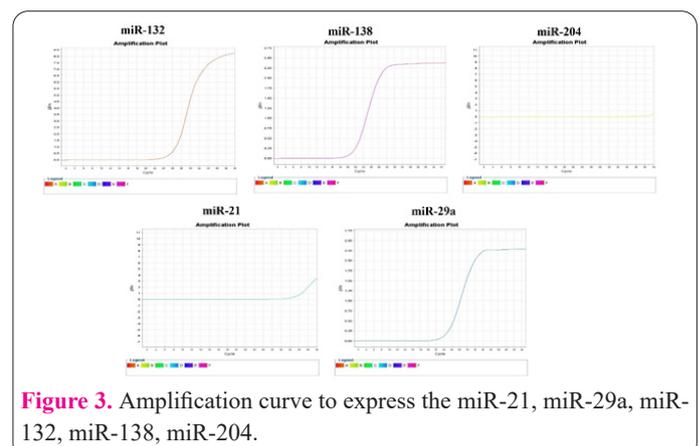


Figure 3. Amplification curve to express the miR-21, miR-29a, miR-132, miR-138, miR-204.

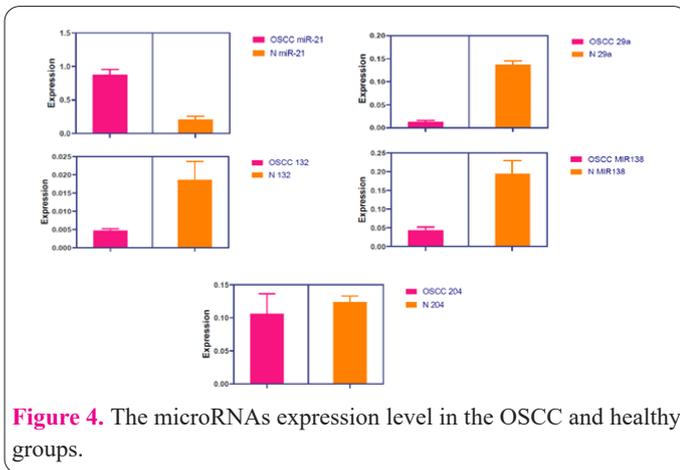


Figure 4. The microRNAs expression level in the OSCC and healthy groups.

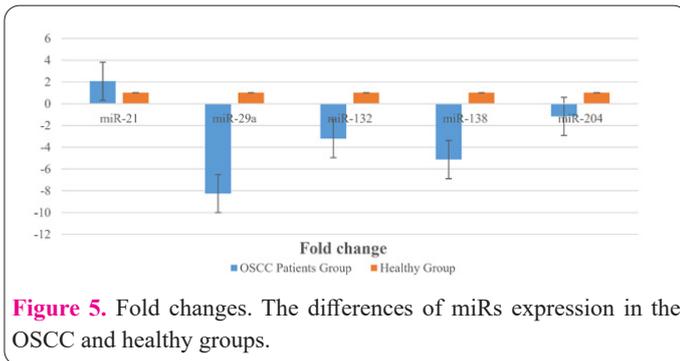


Figure 5. Fold changes. The differences of miRs expression in the OSCC and healthy groups.

markers. Cancer microRNAs and tumor cells can be detected without invasive methods in serum and plasma. As many standard cancer screening methods cannot detect the disease at an early stage, it is considered a critical method to diagnose cancer in a timely fashion by identifying tumor microRNAs released into the bloodstream during the gradual progression of the disease (16). Various types of changes can be observed in miR genes or proteins, such as abnormalities in the co-expression of miR genes or disruptions of the epigenetic regulation of miRs, as well as mutations in miR genes. In tumor cells, miR expression is greatly affected by their location in breaking regions. Moreover, mutations affect miR's binding properties to mRNAs, resulting in insufficient translational processes because of these changed miR:mRNA interactions (18). In normal epithelia, neoplastic mutations cause cell control mechanisms to be lost, cell death to occur, and differentiation to become altered as a result (19). Several miRNAs may affect several cell signaling pathways and contribute to carcinogenesis in human cancers because specific miRNAs are abnormally expressed (20). This mechanism involves miRNAs that are essential for the development of cancer in the early stages, indicating that a lesion has the potential to transform malignantly. As a result of this study, microRNAs were also found to be a high-value biomarker for diagnoses.

The miR-21 expression level in saliva and tumor cells isolated from the lesion was examined in another study conducted in 2021 by Jadhav. Moreover, the microRNA expression level in saliva and tissue cells increased. It was also demonstrated that saliva is a good source of microRNA and that the sample doesn't need to be frozen or stored in any particular way (21). Chen *et al.* researched in 2021 and studied miR-126, miR-155, and miR-21 in patients' plasma. The results showed a remarkable increase in patients' miR-21 and miR-155 expression levels. As a result,

miR-21 is an oncogene suppressing the expression of Bcl-6, PTEN and miR-126 is an exosomal tumor suppressor that decreases the expression of EGFL7 in OSCC (22-24). Researchers conducted the current study by studying miR-21 in plasma samples from patients, which was consistent with previous findings. And also, this microRNA in plasma was also remarkably higher in patients with early OSCC, and this microRNA can be used as a predictor. The reliability of the test is the same in tissue, saliva, and plasma.

They used plasma miR-1290 expression as a diagnostic test in patients with OSCC. Chen *et al.* (2020); examined the diagnostic value of miR-1290 in a patient's plasma. This study examined 70 patients with OSCC and detected miR-1290 expression using PCR. In the OSCC group, plasma miR-1290 expression was remarkably lower than in other groups, according to the research results. Tumor diameter, differentiation grade, miR-1290 plasma expression, and lymph node metastasis are all associated with low miR-1290 levels. Among OSCC patients, clinical stage, lymph node metastatic disease, and miR-1290 levels below 1.14 were independently associated with poor prognoses. It is, therefore, possible to use the miR-1290 expression in plasma to diagnose OSCC patients, as it is significantly reduced in patients with the disease (25). Among patients with oral SCC and healthy controls, soluble CD44 levels were detected in saliva and serum. Researchers found no important difference between groups of patients and control subjects regarding the amount of CD44 soluble in saliva and serum. The number of soluble CD44 in serum and saliva did not differ significantly between sick and healthy subjects. To detect cancer early on, measuring soluble CD44 in serum and saliva can be a valuable method for detecting cancer cells in people (26,27). OSCC tissues overexpressed miR-187, which caused OSCC cells to migrate and form colonies, increasing their oncogenic phenotype. Researchers are able to distinguish between cancer patients and controls using plasma miR-187 levels. After tumor resection, patients with lower miR-187 plasma levels had a better prognosis. It is therefore possible to use miR-187 as a diagnostic marker for OSCC since it plays an oncogenic role in oral carcinogenesis (27,28). A study by Wang *et al.* in 2016 and Tsai *et al.* in 2017 also demonstrated that miR-204 levels decreased in isolated tissues after they measured miRNA expression levels. Researchers have demonstrated a limited amount of this A reduction in the current study, but no significant difference was found. There are several reasons for these differences, including the fact that the two mentioned studies were conducted on East Asian patients, and no information was provided regarding the early stages of the disease, as well as the limited number of samples examined by these researchers, who have only tested tissues (29-35). In the present study, the expression level of mentioned microRNAs was determined in the Iranian genome and plasma samples. According to the OSCC group and the healthy group, miR-21 had a median CT value of 29.68, and miR-29a had a median CT value of 37.54 and 36.46, respectively. Furthermore, miR-132's CT value was 37.71 and 36.40, miR-138's CT value was 35.37 and 31.21, and miR-204's CT value was 36.44 and 36.17. The gene expression variable wasn't normally distributed. A remarkable increase in miR-21 expression levels was detected, while rapid decreases in miR-138, miR-132, and miR-29a expression levels have been observed, and no

significant differences have been observed for miR-204 expression levels.

Conclusion

There was a remarkable difference of the expression level of miR-21 in patients with early OSCC and healthy groups. There were significant differences between the expression level of miR-132, and miR-138, miR-29a. And also, microRNA-204 did not show any significant variations, and these microRNAs can also be used as blood biomarkers for early diagnosis.

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Conflicts of interest

There are no conflicts of interest.

Author's contribution

Designed the study: Elahe Tahmasebi, Lotfollah Kamali Hakim, Mostafa Khafaei. **Performed experiments, data analysis and results:** Elahe Tahmasebi, Lotfollah Kamali Hakim, Mohsen Yazdaniyan, Amirali Asadi, Ata Garajei, Sayna Nezaminia, Amir Mohammad Arabi Daredor, Mostafa Khafaei, Mahmood Tavallaie. **Supervised, directed and managed the study:** Elahe Tahmasebi, Mohsen Yazdaniyan, Mostafa Khafaei, Mahmood Tavallaie. **Approved of the version to be published:** Elahe Tahmasebi, Lotfollah Kamali Hakim, Mohsen Yazdaniyan, Amirali Asadi, Ata Garajei, Sayna Nezaminia, Amir Mohammad Arabi Daredor, Mostafa Khafaei, Mahmood Tavallaie.

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