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Evaluation of circulating serum 3 types of microRNA as biomarkers of oral squamous cell carcinoma; A pilot study

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Abstract

Introduction: The microRNAs are molecules which have important biologic role and play key point in cancers. The aim of present study was to determine the miR-21, miR-24, and miR-29a expression in serum of patients with oral squamous cell carcinoma.

Materials and methods: Blood samples were obtained from 40 patients (20 in cases and 20 in control group) to determine the miR-21, miR-24, and miR-29a expressions by using real-time PCR and ΔCT.

Results: Mean miR-29a was −2.28 ± 2.15 and 5.61 ± 2.38 in case and control groups, respectively. The miR-21 was 6.90 ± 3.86 and −0.88 ± 2.31 in case and control groups, respectively. According to the results, miR-24 was 2.13 ± 2.89 and −0.35 ± 2.44 in case and control, respectively. A significant difference was observed on miR-21, miR-24, and miR-29a between two groups ($P < .05$). The results obtained by t test showed miR-21 and miR-24 were higher and miR-29a was lower in plasma of oral squamous cell carcinoma patients and this differences were significant ($P < .05$).

Conclusion: These results suggested miR-21, miR-24, and miR-29a in serum of patients with oral squamous cell carcinoma comparing with normal group can be used as potent markers for carcinoma detection and also may be a potentially therapeutic approach in the future. More longitudinal studies with larger samples are necessary to confirm these findings.

KEYWORDS

microRNA, miR-21, miR-24, miR-29a, oral, squamous cell carcinoma

1 | INTRODUCTION

More than 90% of the malignancies in the oral cavity are belonged to oral squamous cell carcinoma (OSCC) which is characterized by spreading to the cervical lymph nodes.[1] Molecular studies have demonstrated novel biomarkers for evaluation in diagnostic, therapeutic, and prognostic levels to improve survival rate of patients of OSCC especially in early stages.[2,3] The expression patterns of miRNAs (miRs) in oral squamous cell carcinoma represent new directions in the search of oral carcinogenesis.[4] Although there are many studies about miRs in serum, plasma and urine in different type of carcinomas such as OSCC, but circulating miRs, may play an important role as diagnostic or prognostic biomarkers in human carcinomas.[5] Also recently, circulating miRs have been described as biomarkers for head and neck cancers in multiple anatomical sites including the skin, the oral cavity, nasopharynx, larynx, and salivary glands.[6] The miRs are small, single-stranded, non-coding RNA molecules that program many intracellular processes including cell differentiation,

progression of cell cycle, and beginning of apoptosis cascade.[5] These magic molecules can also play the role of oncogenes in progression of cancer processes or tumor suppressor genes.[3,7] The miR-21 is an oncogene that targets many genes such as tropomyosin-1, phosphate, and tensin homologue and programmed cell death-4.[8] The miR-24 is a multifunctional biomarker which regulates a variety of biological processes. One of its important roles is regulating cell apoptosis and proliferation via targeting inhibitors of cyclin-dependent kinases such as CDKN1B and CDKN2A.[9] Over and down expressions of the miR-29 family miRNAs (including mir-29a) have been reported in several types of carcinomas.[10] It seems that the expression level of miR29 family members varies based on the type of cancer. In the present study, we analyzed circulating serum miR-21, miR-24, and miR-29a expression in patients with OSCC in comparison with healthy control individuals to identify the use of these biomarkers as diagnostic tool for effective detection of OSCC. Based on our best knowledge, this is the first study that evaluates miR-21, miR-24, and miR-29a simultaneously in patients with OSCC. The aim of our study was demonstration of some serum miRNAs to serve for difference between patients with OSCC and healthy persons especially in early stage.

2 | METHODS AND MATERIALS

2.1 | Sample collection

The present study included 20 patients with OSCC and 20 healthy controls (Table 1). The blood samples were derived from patients with OSCC referred to Oral and Maxillofacial Surgery Department of Shariati Hospital, Tehran University of Medical Sciences (TUMS). The blood samples of healthy controls were derived from healthy individuals who hospitalized for esthetic or traumatic fracture surgical treatments. The exclusion criteria in this study were as follows:

- Patients of SCC who have or had in past any other malignancies or history of head and neck radio or chemotherapy.
- Patients who had any known immunodeficiency disorder such as AIDS.
- Patients who were in end stage of disease and was not operable.
- Patients who had immune or autoimmune diseases.

The written informed consent was obtained from all patients. This study was approved by Ethics committee of TUMS #IR.TUMS.DENTISTRY.REC.1396.2165.

2.2 | Serum samples storage and total RNA extraction

Peripheral blood samples were obtained before any therapeutic intervention including surgery or pre-operative radiation. All samples were collected in clot stimulator tube. The basic clinical information was obtained from all participants. Tumor staging has been done according to American Joint Committee on Cancer (AJCC).[6,7] The blood was kept for 45 minutes to allow clotting for serum separation, and thereafter, it was processed according to Mircury exiqon (Denmark) protocol. The blood samples were centrifuged at 4°C in 2500 x*g*, for 15 minutes. (REMI). The fluid was re-centrifuged at 4°C at full spin for 15 minutes to remove other contaminants like erythrocytes. In addition by this way, better utility of miR yielded for further processing of total RNA isolation. The serum was stored at −70°C until processing for total RNA isolation. According to manufacturer's protocol, total RNA was extracted using Mircury exiqon (Denmark) from serum

TABLE 1 The clinicopathologic characteristics in OSCC patients and control group

Clinical characteristics	Average N° (Range/%) in OSCC patients	Average N° (Range/%) in control group
Age	46.60 ± 10.69	47.10 ± 17.66
Gender		
Male	14 (70%)	14 (70%)
Female	6 (30%)	6 (30%)
Smoking condition		
Yes	10 (50%)	6 (30%)
No	10 (50%)	14 (70%)
Recurrence		
Yes	7 (35%)	–
No	13 (65%)	–
Metastasis		
Yes	12 (60%)	–
No	8 (40%)	–
Neck dissection		
Yes	8 (40%)	–
No	12 (60%)	–
Total	20 (100%)	20 (100%)

TABLE 2 Distribution of expression level of miR-21, miR-24, and miR-29a

	Mean ± SE in OSCC patients (n = 20)	Mean ± SE in control group (n = 20)
miR-21	−0.88 ± 2.33	6.90 ± 3.89
miR-24	−0.35 ± 2.44	2.13 ± 2.89
miR-29a	−2.28 ± 2.15	5.61 ± 2.38

Abbreviation: SE, standard error.

samples. For this, the miRNeasy mini Kits Mircury exiqon (Denmark) and miRNeasy serum Mircury exiqon (Denmark) were used to extract miRs from serum samples. In the other hand, miRNeasy procedures decreased the potential of contamination with phenols or salt those interfere with other processing. According to manufacturer's protocol, optical density for RNA extraction was 260-280 ratio.

2.3 | cDNA synthesis

In this study, after normalization of all extracted to 2 μg, cDNA synthesis was done by using cDNA syntheses kit (Universal, Exiqon). Synthesized cDNAs from the total RNAstem-loop were used in real-time RT-PCR. The sequences were as follows: 5′-GTCGTATCCAGTG CAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3′(miR-21), 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGC AATTGCACTGGA TACGACAAACTGAT-3′ (miR-29a), GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGAT ACGACCTGTTC (miR24). Reverse transcriptase reactions contained the following reagents: 2 μg RNA sample, 50 nmol/L stem-loop RT primer, 2 × RT buffer, 0.5 mmol/L each of dNTP, and 4 U/μL M-MLV reverse transcriptase.Reactions (20 μL) were incubated in a PCR System at 37°C for 50 minutes and 85°C for 5 minutes. The samples were then held at 4°C.

2.4 | Real-time PCR

Real-time PCR was performed using SYBR Green PCR Master Mix (Pars Genum, Iran) on real-time PCR instrument according to manufacturer's instruction. Each reaction was done in a volume of 20 μL, containing 50 ng cDNA, μL universal primer, and 10pmol each primers along with 10 μL 2× QuantiTect SYBR Green PCR Master Mix. The PCR amplification reaction consisted of de-naturation at 95°C for 5 seconds followed by 40 cycles at 62°C for 20 seconds and 72°C for 30 seconds. All reactions were carried out in triplicate. The comparative Ct method was applied to analyze the differences in each group in expression levels. The expression levels of miR-21, miR-24, and miR-29a were normalized by using the endogenous control mir191 (Applied Biosystems).

2.5 | Statistical analysis

All statistical analysis was performed using SPSS 21.0 Software. To compare miRNAs serum expression level findings, *t* test was used and differences with a *P*-value < .05 considered as statistically significant. For evaluation of demographic information, multiple liner regression test was done (Enter Method). Due to use Rest Software, the negative mark means increasing in gene expression. Gene Runner primer design software was used.

3 | RESULTS

3.1 | Patient description

In total, 40 participants including 20 patients with OSCC and 20 healthy individuals were enrolled in our study. The mean age of the control and case groups was 47.10 ± 17.66 and 46.60 ± 10.69, respectively. Fourteen (70%) men and 6 (30%) women were in both groups. Among the evaluating risk factors in patients with OSCC, 10 (50%) were positive for smoking. There are 6 (30%) smokers in control group. Unfortunately, we could not evaluate alcohol consumption because of deficiency of documented information. Recurrence in 7 (35%) patients and metastasis in 12 (60%) patients were seen in OSCC group. Neck dissection was done in 8 (40%) patients with OSCC. Demographics parameters of OSCC and control samples are listed in Table 1.

3.2 | Expression levels of serum miRNAs in OSCC patients

The distribution level of miRNAs and regression coefficient has been shown in Tables 2 and 3. A significant correlation was observed on miR-21, miR-24, and miR-29a between two groups ($P < .05$). The mean levels of serum miR-21 and miR-24 in OSCC cases were significantly higher than the healthy control groups versus the level of serum miR-29a was lower than the healthy group (all $P < .001$).

3.3 | miR-21

It was observed that mean fold increase in OSCC cases was more significant than control group ($P < .001$). The miR-21 was −0.88 ± 2.31 and 6.90 ± 3.86 in case and control group. A significant correlation was observed on miR-21 between 2 groups ($P < .05$). The regression coefficient for miR-21, age, gender, and smoking history was −7.64, 0.03, −1.32, and 0.76, respectively. A significant correlation was found with age, gender, and smoking history in the patients with OSCC (data not shown).

3.4 | miR-24

It was observed that mean fold increase in OSCC cases was more significant than control group ($P < .001$). According to the results, miR-24 was −0.35 ± 2.44 and 2.13 ± 2.89 in OSCC and control group. A significant correlation was observed on miR-24 between 2 groups ($P < .05$). The regression coefficient for miR-24, age, gender, and smoking history was −2.17, −0.02, −0.62, and 1.59, respectively. A significant correlation was found with age, gender, and smoking history in the patients with OSCC (data not shown).

TABLE 3 Regression coefficient of expression level of miR-21, miR-24, and miR-29a

	Regression coefficient	95% CI		*P* value
		Lower limit	Upper limit	
miR-21	−7.77	−9.83	−5.72	<.001
miR-24	−2.48	−4.20	−0.77	<.001
miR-29a	−7.89	−9.34	−6.44	<.001

Abbreviation: CI, confidence interval.

TABLE 4 Sensitivity and specificity of serum miRNAs as potential markers for distinguishing OSCC patients from other healthy groups

	Sensitivity (95% CI)	Specificity (95% CI)
miR-21	95 (76.39-99.11)	95 (76.39-99.11)
miR-24	80 (58.4-91.93)	70 (48.1-85.45)
miR-29a	100 (83.89-100)	100 (83.89-100)

Abbreviation: CI, confidence interval.

3.5 | miR-29a

It was observed that mean fold decrease in OSCC cases was more significant than control group ($P < .001$). Mean miR-29a was −2.28 ± 2.15 and 5.61 ± 2.38 in patients with OSCC and control group. A significant correlation was observed on miR-29a between 2 groups ($P < .05$). The regression coefficient for miR-29a, age, gender, and smoking history was −8.02, −0.02, 0.71, and −0.72, respectively. A significant correlation was found with age, gender, and smoking history in the patients with OSCC (data not shown).

3.6 | Evaluation of serum miRNAs as a potential marker

This analyze was performed to evaluate whether the serum miRNAs can be used as potential diagnostic markers for OSCC. After comparison of expression levels of 3 miRNAs between the patients with OSCC and the healthy group, the sensitivity and specificity for each miRNA in OSCC diagnosis were calculated with using the cut-off values and Wilson Score Interval method (Table 4). The result revealed that the levels of serum miR-21, miR-24, and miR-29a were potential markers for discriminating patients with OSCC from healthy donors. The sensitivity and specificity for miR-21 were 95 and 95, for miR-24 were 80 and 70, respectively, and for miR-29a were 100 and 100. Based on these findings, the serum levels of 3 miRNAs (miR-21, miR-24, and miR-29a) could serve as potential biomarkers to distinguish patients with OSCC from healthy patients.

4 | DISCUSSION

It seems the gap between laboratory studies and clinical practice grows up every day and providing a breakthrough in applying the tumor markers in clinical practice is very important.

Currently, there are no specific tumor markers for OSCC in clinical practice; therefore, it is hoped that some tumor biomarkers including novel miRNAs markers can be used as potential biomarkers to distinguish patients with OSCC from healthy individuals.[11] In the present study, we highlighted the potential application of serum miRNAs with high sensitivity, specificity, and reliability as biomarkers of OSCC in the clinical practice. MicroRNAs, a length of 19-22 nucleotides, are a series of endogenous non-coding RNAs. They function in regulating gene expression and also RNA silencing in post-transcriptional level.[12] Previous studies reported that deregulation of miRNAs is introduced in initiation and progression of various types of cancers.[13]

In addition, circulating miRNAs are stably preserved and measurable in blood which can serve as reliable biomarkers for the diagnosis or prognosis for some carcinomas [11,13] including oral squamous cell carcinoma [5] and also can suggest a field effect in oral cancer.[1] We suggested that serum miR-21, miR-24, and miR-29a, particularly miR-21 and miR-29a, could be good tumor biomarkers with stable sensitivity and specificity for diagnostic monitoring.

Many previous studies have reported miRNAs may act as oncogenes or tumor suppressor genes.[5,14] In the other hand, tumoral tissues demonstrate significantly different expression pattern of miRNAs in comparison with normal tissues.[15,16]

Up-regulation of miR-21 has been reported in many carcinomas as it up-regulated in patients with OSCC in the present study. On the other hand, it has been reported that expression level of miR-21 is significantly associated with development of tumor.[17] In a recent review study, miR-21 presented as a potential prognostic marker and associated with resistance to chemotherapy significantly.[18] Also, miR-21 can induce tumor cell proliferation and invasion by targeting PTEN and PCD4.[18] Additionally, in the present study significant relationship was observed between expression of miR-21 and demographical parameters including age, gender, and smoking.

We reported also the up-regulation of miR-24 in patients with OSCC. This finding is in agreement with the other observation based on up-regulation of miR-24 in OSCC.[19,20] However, miR-24 has been shown a different effect in laryngeal SCC.[21,22] However, its real role in tumorigenesis is not clear. Zhao reported that high miR-24 level in tongue SCC is negatively correlated with PTEN expression and is associated with poor prognosis.[19] However, Baghaei reported that PTEN expression may be regulated via miR-26b in OSCC.[23] Zheng described that miR-24 induces cell survival and

resistance to cisplatin through targeting PTEN-Akt pathway in tongue squamous cell carcinoma.[20] Zheng claimed deregulation of miR-24 is a recurrent event in human tongue squamous cell carcinoma which is associated with tumor progression.[20] Another target for miR-24 is one tumor suppressor gene of F-box and WD-40 domain protein 7 (FBXW7). Zhao reported that overexpression of potential biomarker of miR-24 is correlated with the proliferation, migration, and invasion of tongue SCC cells in vitro through regulation of functional target FBXW7.[24] It has been reported that miR-24 facilitates OSCC cells growth by targeting p57.[18] Further studies are warranted to discover the real mechanisms of miR-24 in OSCC. In addition, in the present study significant relationship was found between expression of miR-24 and demographical parameters including age, gender, and smoking.

Many previous studies showed that miR-29 family members such as miR-29a, miR-29b, and miR-29c are down-regulated in several types of cancers.[25,26] In addition, some studies have showed that all members of the miR-29 family inhibit migration and invasion of tumoral cells.[27,28] Several studies have demonstrated the down-regulation of the miR-29 family in head and neck squamous cell carcinoma like other cancers, and showed the antitumor functions of them through targeting several oncogenic genes.[28,29] Kinoshita found that down-regulation of miR-29 family with overexpression of laminin (LAMC2) and integrin (ITGA6) in head and neck squamous cell carcinoma cells lead to cell migration and invasion.[28] In this present study, the level of serum miR-29a of patients with OSCC was significantly lower than in the healthy group which is in agreement with the other previous studies based on down-regulation of miR-29a in patients with OSCC versus healthy individuals. Like miR-24 and miR-21, there was significant relationship between expression of miR-29a and demographical parameters in present study.

Considering that the overall survival of patients with OSCC is very poor, screening for novel diagnostic biomarkers seems be an urgent need in confronting with OSCC patients. In comparison with other bodily fluids, blood is more applicable in general as it is easily attained in a non-invasive approachable manner and can be stored for long time. Thus, since 2008, circulating miRNAs are known as diagnostic biomarkers and important approaches for the blood-based detection of human cancer.[30]

The measurement of miRNAs in serum can help to earlier detection of cancers including OSCC. Circulating miRNAs might serve as biomarkers for risk of OSCC development, prognosis, and also response to treatment. Despite recent advances in the field of miRNAs, therapeutic use for miRNAs requires further studies based on a suitable insight of real mechanisms of them in OSCC.

5 | CONCLUSION

In summary, the results of this study clearly described that serum miR-21 and mir-24 level is significantly higher and mir-29a expression level is significantly lower in patients with OSCC than healthy individuals. Also the demographical parameters including age, gender, and smoking have shown a significant positive correlation with expression of these miRNAs in oral cancer. This pattern of serum miRNAs could potentially use as a non-invasive and efficiency biomarker with high sensitivity and specificity for OSCC diagnosis. Larger cohorts of participants are necessary to explore the real mechanisms and the clinical application of three serum miRNAs.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

RESEARCH INVOLVING ANIMAL OR HUMAN PARTICIPANTS

This study does not contain any intervention with human or animals participants performed by any of the authors.

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