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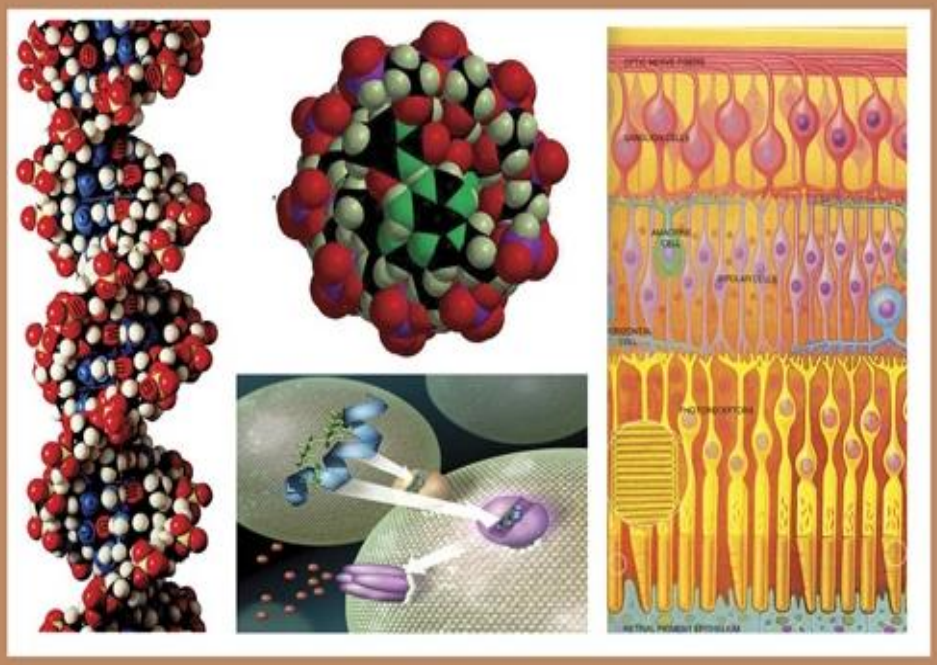

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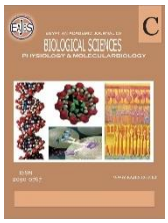

Differential Expression of miR-589-5p, miR-569 and *c-Fos* gene in Oral Squamous Cell Carcinoma

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most prevalent cancer among the Head and Neck Squamous cell carcinomas (HNSCC) group. Most OSCC cases are diagnosed at advanced stages, resulting in poor survival and a high mortality rate. This study aimed to investigate the changes in expression levels of miR-589-5p, miR-569, and *c-Fos* (as a target gene) in tumor and adjacent normal tissues from OSCC patients. Also, the association between the *c-Fos* gene expression and several clinicopathological factors was evaluated. **Methods:** *c-Fos* was predicted as a potential target gene of miR-589-5p and miR-569 using the Mirwalk and miRDB bioinformatic algorithms. The expression levels of these miRNAs and *c-Fos* target gene in 30 OSCC tissues compared to their adjacent-normal tissue samples were analyzed by quantitative real-time PCR. In addition, the potential diagnostic values of miR-589-5p and miR-569 in OSCC as risk factors of carcinogenesis were assessed by ROC curve analysis. **Results:** *c-Fos* expression was significantly increased, while miR-589-5p and miR-569 gene expressions were decreased in tumor tissues compared to normal tissues. The increased *c-Fos* expression was also significantly correlated with tumor necrosis (p=0.034). We found that downregulation of miR-589-5p and miR-569 was negatively correlated with overexpression of the *c-Fos* target gene. The area under the curve (AUC) of mir-589-5p and mir-569 was 0.865 and 0.591, respectively. **Conclusion:** miR-589-5p may serve as a potent diagnostic and prognostic biomarkers for OSCC patients and be a potential approach to early diagnosis or prognosis of OSCC in the future. *c-Fos* overexpression may have a role in OSCC progression and necrosis.

INTRODUCTION

Oral cancer is a group of malignant neoplasms developing on the lip or oral cavity, in which more than 95% of all cancers are oral squamous cell carcinomas (OSCCs) (Awan *et al*, 2016). OSCC is the most prevalent cancer among the Head and Neck Squamous cell carcinomas (HNSCC) group (48% of cases) (Irani, 2020) and the sixth most common cancer in the world (Rivera, 2015; Borse *et al*., 2020). According to the International Agency for Research on Cancer in 2020, GLOBOCAN has estimated 377,713 new cases and 177,757 deaths related to lip and oral cavity cancer (Sung *et al*., 2020). The incidence pattern of OSCC is geographically different, with a higher incidence rate in South Asia, South Central Asia, and the Pacific Islands.

For example, in Iran, as a part of Southern Asia, prevalence is 20 to 36.3 in each 100 thousand people (Maleki *et al*., 2015; Anwar *et al*., 2020). Despite advances in screening and diagnosis of OSCC, most patients are not diagnosed until cancer has reached an advanced stage, such as stage III or IV, which is subsequently associated with poor survival and a high mortality rate. Therefore, it is crucial to keep finding new approaches for early oral cancer diagnosis, especially in high-risk communities (Awan *et al*, 2016; Irani, 2020).

In recent years, one new area of research has been to evaluate the role of various microRNAs (miRNAs) in carcinogenic mechanisms and their potential in clinical applications such as diagnosis. miRNAs are small single-stranded, non-coding RNA molecules (21 to 23 nucleotides), which regulate the expression of their target genes (messenger RNAs) through a mechanism named RNA interference (RNAi) at transcriptional and or post-transcriptional levels (Annese *et al*., 2020). Therefore, any aberration in the expression pattern of miRNAs can lead to dysregulation of target genes, resulting in induction of pathogenic processes such as inflammation, invasion, metastasis, and chemoresistance (Shenouda *et al*., 2009). Furthermore, the role of miRNAs as oncogenes or tumor suppressors has been proven in various types of human cancers. For example, gene expression analysis of 72 miRNAs in tissue samples of oral cancer revealed a decreased expression of three miRNAs (miR-125a, miR-184, and miR-16) and an increased expression of one miRNA (miR-96) compared to normal tissues, indicating their potential involvement in oral tumorigenesis (Santhi *et al*., 2013).

c-Fos is a proto-oncogene and a transcription factor belonging to the Fos family. The members of this family encode leucine zipper proteins that can dimerize with proteins of the JUN family to form the transcription factor activating protein 1 (AP-1). c-Fos, as a member of the AP-1 family, involves primarily in signal transduction, cell differentiation, and proliferation (Milde-Langosch, 2005). The presence of c-Fos has been reported in various types of cancers, including HNSCC. In OSCC, it has been shown that c-Fos stimulates cell invasion and migration via the CD44 pathway (Dong *et al*., 2015). c-Fos is regulated through various miRNAs, such as miRNA-569 and miR-589-5p. MiRNA-569 acts as a potential tumor suppressor gene in lung cancer by downregulating the *Fos* gene. Aberrant expression of this microRNA led to cell proliferation and migration and inhibition of apoptosis in lung cancer, suggesting its potential as a diagnostic biomarker (Zheng *et al*., 2018).

Furthermore, an increased expression of miR-569 is associated with tumor growth and metastasis *in vivo* (Chaluvally-Raghavan *et al*., 2014). Several studies have examined the biological effect of miR-589 in several human cancers. As an oncogene, MiR-589 induced invasion and metastasis in gastric cancer cells through an atypical regulatory feedback loop called miR-589-LIFR-PI3K/AKT-c-Jun (Leukemia inhibitory factor receptor), which suggests miR-589 as a potential biomarker and or therapeutic target for gastric cancer (Zhang *et al*., 2018). Also, the potential role of miR-589 as a new prognostic biomarker and a therapeutic target against non-small cell lung cancer has been reported (Liu *et al*., 2017). In the present study, we selected miR-589-5p and miR-569 from bioinformatics databases (Mirwalk (http://mirwalk.uni-hd.de) and miRDB (http://mirdb.org)), and c-Fos was predicted as the targeted gene. To date, no study has evaluated the gene expression levels of miR-589-5p and miR-569 in Iranian patients with oral cancer. Therefore, this study aimed to investigate

the changes in expression levels of miR-589-5p, miR-569, and *c-Fos* (as a target gene) in tumor and adjacent normal tissues from OSCC patients. Finally, the association between the *c-Fos* gene expression and several clinicopathological factors was evaluated to assess its role in the malignancy of OSCC.

MATERIALS AND METHODS

Patients and Sample Collection:

This study was approved by the ethics committee of Islamic Azad University, East Tehran Branch, Tehran, Iran (IR.IAU.ET.REC.1400.036). The samples consisted of 30 pairs of tumor and adjacent normal tissues from patients diagnosed with oral cancer between October 2020 and January 2021 in Imam Khomeini Hospital, Tehran, Iran. Patients who received adjuvant therapies, including chemotherapy and radiotherapy, were excluded from the study. Written informed consent was obtained from the patients, and the clinicopathological data related to OSCC patients are shown in Table 2. All samples were approved by an orthodontic specialist and a pathologist and are available in Tumor Bank of Cancer Institute (Imam Khomeini Hospital, Tehran, Iran). The fresh tissue samples were frozen immediately after surgery and stored in a nitrogen tank until RNA extraction.

Reverse Transcription and Quantitative real-time PCR (RT-qPCR):

Total RNA was extracted using TRIZOL reagent (Invitrogen, Sigma, USA), following the manufacturer's instructions. Extracted RNAs were quantified and qualified using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and electrophoresed in 1.5% agarose gel, respectively. Reverse transcription of total extracted RNA to obtain complementary DNA (cDNA) was performed using BioFACT cDNA Synthesis kit (Daejeon, South Korea). In addition, cDNA for miRNAs was synthesized using appropriate stem-loop RT primers and the MiR-Amp kit (Pars Genome, Iran). Quantitative real-time PCR analysis was performed using the SYBR Green RT-PCR Kit (BioFact, Daejeon, South Korea) on a Light Cycler TM 96 (Roche). PCR profile included one cycle of 2 min/95 °C for initial denaturation, followed by 40 cycles of 15 s/95 °C for denaturation, 30 s/60 °C for annealing, and 20s/72 °C for elongation. Also, melting curve analysis was carried out to assess the existence of primer-dimers and non-specific products. U6 and ACTB were used as housekeeping genes to normalize the expression of *c-Fos* and miRNAs, respectively. Finally, Real-time PCR data were analyzed by the comparative threshold cycle (C_t) method. Primers were designed using OligoAnalyzer and Primer3plus, analyzed for optimal properties by BLAST program, and synthesized by BIONEER (Daejeon, South Korea). The primer sequences are summarized in Table 1.

Table 1. Real-time quantitative PCR primers.

Genes	5'-3' sequence
c-Fos	Forward 5´-CAAGCGGAGACAGACCAACT-3´ Reverse 5´-AGTCAGATCAAGGGAAGCCA-3´
miR-589-5p	Forward 5´-CGAGGTCAGCGTGATTTCATGG-3´ Reverse 5´-TGTGTCCAAGTCCCAGCCAGAG-3´
miR-569	Forward 5´-AGACTGCTGAGTTAATGAATCCTG-3´ Reverse 5´-TATGGTTGTTCACGACTCCTTC-3´
ACTB	Forward 5´-GATCAAGATCATTGCTCCTCCTG-3´ Reverse 5´-CTAGAAGCATTTGCGGTGGAC-3´
U6	´Forward 5´- CTCGCTTCGGCAGCACA-3 ´Reverse 5´- AGAGCAGGGTCCGAGGT-3

Statistical Analysis:

Results are presented as mean ± SEM of three identical experiments made in triplicates. The data were analyzed using GraphPad Prism software 9.0.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). The One-Sample Kolmogorov-Smirnov Test was used to assess the normal distribution of sample data. The associations between miR-589-5p, miR-569, and *c-Fos* levels and clinicopathological parameters of OSCC patients were assessed using independent-sample Kruskal-Wallis tests. Pearson correlation analysis was performed to determine the correlation between miR-589-5p, miR-569, and *c-Fos* expression. In addition, the one-way analysis of variance (ANOVA) was used to evaluate the differences in *c-Fos* expression levels between different tumor sizes. Finally, the receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value. A p-value less than 0.05 (≤0.05) was considered to indicate a statistically significant.

RESULTS

miR-589-5p and miR-569 Expression Were Downregulated in OSCC:

The expression patterns of miR-589-5p and miR-569 in 30 paired OSCC tissues and adjacent normal oral tissues were evaluated by RT-qPCR analysis. As shown in Figures 1 A and B, the expression levels of both miR-589-5p and miR-569 were decreased (~6-folds and ~2-folds, respectively) in OSCC tissues compared to normal tissues (p = 0.2084 and p=0.0019, respectively).

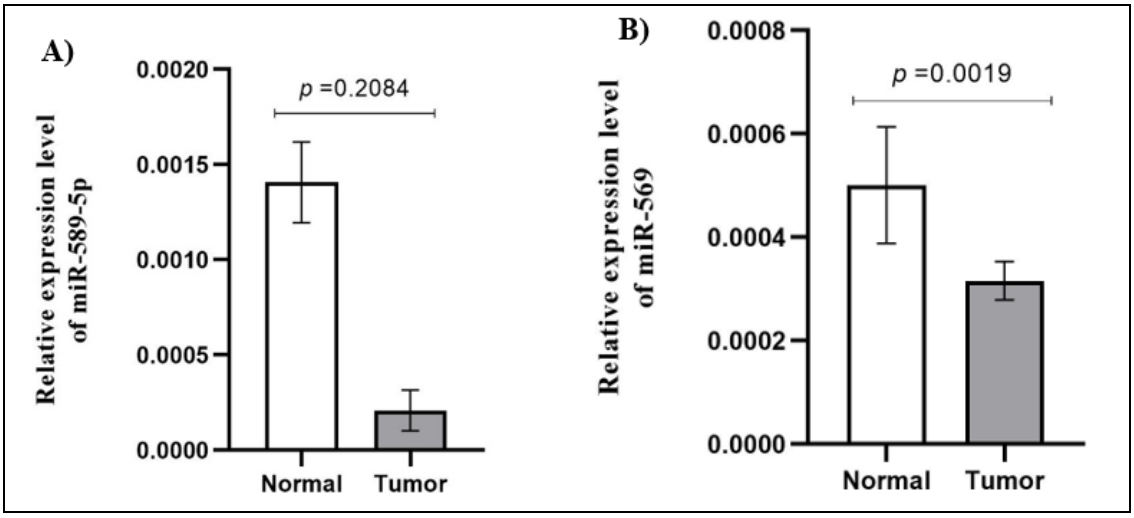

Fig. 1. Quantitative RT-PCR analysis of miR-589-5p and miR-569 expressions in OSCC tissues and adjacent normal tissues (n=30) (A and B). Transcript levels were normalized to U6 expression. Data are presented as means ± SD

***c-Fos* gene Expression Was Upregulated in OSCC:**

In this study, the expression of *c-Fos* as a potential target for miR-589-5p and miR-569 was evaluated using RT-qPCR analysis in 30 paired OSCC tissues and adjacent normal oral tissues. We used Mirwalk and miRDB algorithms to find the putative co-targets gene of miR-589-5p and miR-569 in OSCC. Then, based on the online bioinformatics databases, *c-Fos* was selected as a potential direct target for corresponding miRNAs. The results showed that *c-Fos* expression was significantly increased (~2-folds) in tumor tissues compared to adjacent normal oral tissues (p <0.0001) (Fig. 2).

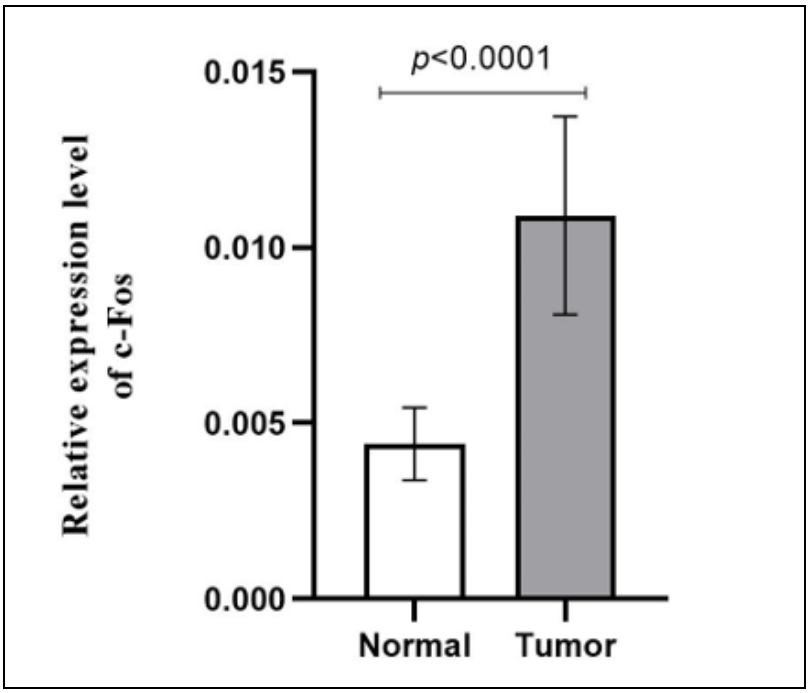

Fig. 2. Quantitative RT-PCR analysis of *c-Fos* expression in OSCC tissues and adjacent normal tissues (n=30). Transcript levels were normalized to ACTB expression. Data are presented as means ± SD.

The Association between *c-Fos* Expression and Clinicopathological Characteristics:

As shown in Table 2, the association between *c-Fos* expression levels and different clinicopathological features of patients were assessed to better understand the potential role of *c-Fos* in the malignancy of OSCC. The results revealed that *c-Fos* expression was remarkably associated with tumor necrosis (p=0.034). Considering the necrosis presence, 23% of patients (n=7) were positive, and 76% (n=23) were negative. The level of *c-Fos* expression was increased in all patients, but given the number of patients with necrosis, the presence of necrosis was significantly associated with *c-Fos* upregulation (p<0.05). Other clinicopathological features including gender (p=0.210), age (p=0.890), tumor size (p=0.895), pathological grading (p=0.604), clinical-stage (p=0.455), lymph node status (p=0.895), vascular invasion (p=0.311), perineural invasion (p=0.081), depth invasion (p=0.614), metastasis (p=0.584), family history (p=0.499) and smoking status (p=0.830) had no statistically significant association with the increased expression of *c-Fos*.

Table 2. Association between *c-Fos* expression and the clinicopathological features of OSCC patients**.**

Clinicopathological Characteristic	Total cases (n=30)	*p*
Gender		0.210
Female	7	
Male	23	
Age (years)		0.890
>40	27	
<40	2	
Tumor Size (cm)		0.895
<2	5	
2-5	14	
>5	11	
Pathological grading		0.604
I	17	
II	13	
Clinical Stage		0.455
I	3	
II	3	
III	7	
IV	17	
Lymph node metastasis		0.895
Yes	5	
No	24	
Unknown	1	
Vascular invasion		0.311
Yes	6	
No	24	
Perineural invasion		0.081
Yes	11	
No	19	
Depth invasion		0.614
Yes	9	
No	21	
Necrosis presence		0.034
Yes	7	
No	23	
Metastasis		0.584
Yes	1	
No	29	
Family history		0.499
Yes	6	
No	24	
Smoking status		0.830
Non-smokers	22	
Smokers	7	
Ex-smokers	1	

OSCC: Oral squamous cell carcinoma

The Correlation between *c-Fos* Expression and miR-589-5p, miR-569 in OSCC Patients:

The correlation between miR-589-5p and miR-569 levels and *c-Fos* expression in OSCC was assessed using a correlation analysis by Pearson method. We observed an inverse correlation between downregulation of miR-589-5p and miR-569 in OSCC with overexpression of the *c-Fos* target gene ($r=-0.0526$, $p= 0.7825$ and $r=-0.1026$, $p= 0.5897$, (Figs. 3 A and B).

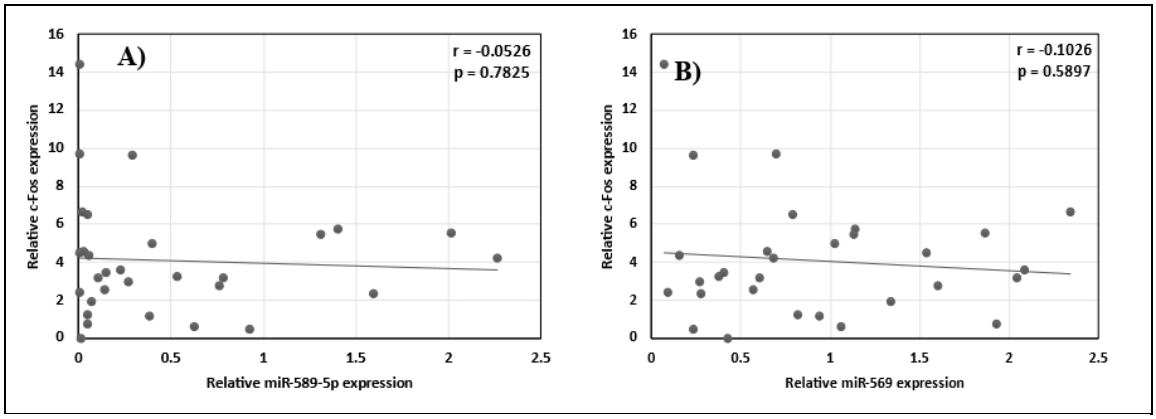

Fig. 3. Pearson's correlation analysis between c-Fos mRNA expression and miR-589-5p (A) and miR-569 (B) levels in OSCC patients. Data are presented as means ± SE. No significant correlation was found between the expression of *c-Fos*, miRNAs in OSCC patients' tissues. **P* < 0.05.

Potential Diagnostic Values of miR-589-5p and miR-569 in OSCC:

The potential diagnostic values of mir-589-5p and mir-569 for OSCC were evaluated by ROC curve analysis. The area under the curve (AUC) of mir-589-5p and mir-569 was 0.865 (95% CI: 0.769–0.961; *p* <0.0001) and 0.591 (95% CI: 0.445–0.973; *p*=0.225), respectively (Figs. 4A and B). The ROC curve analysis showed a good predictive value of mir-589-5p for OSCC patients.

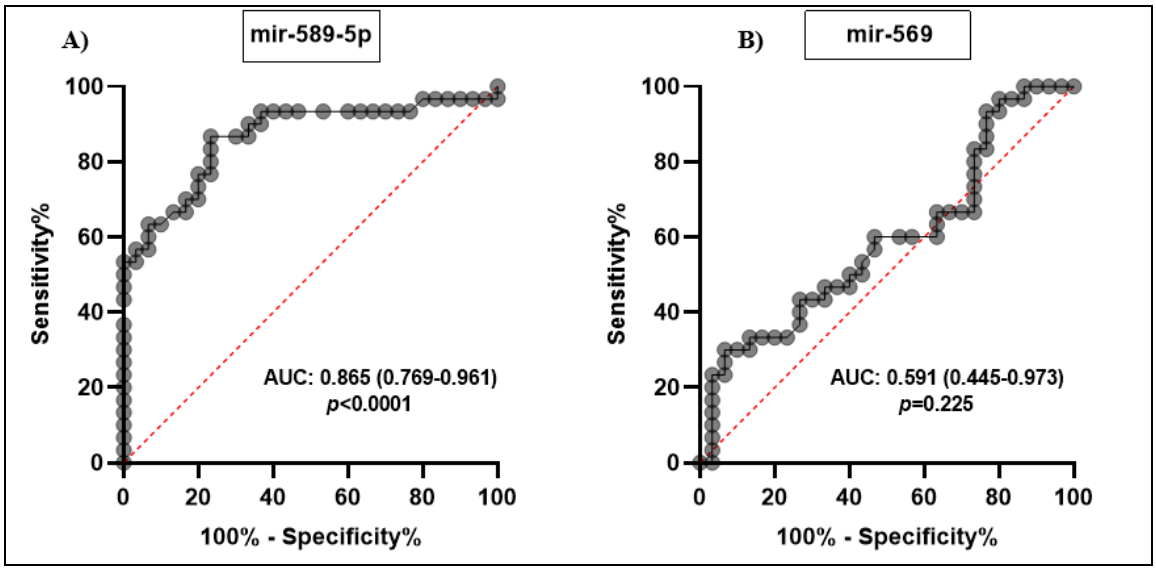

Fig. 4. ROC curve analyses related to the expression of miR-589-5p (A), miR-569 (B) to discriminate patients with OSCC from healthy controls.

DISCUSSION

The prevalence of OSCC is increasing worldwide, and early detection is one of the major challenges of the global health system. Currently, ongoing efforts have been emerging to provide new diagnostic methods, such as biomarkers, to improve the prognosis and the effectiveness of treatment for oral cancers (Irani, 2020).

MicroRNAs are regulators of various cellular processes such as division,

differentiation, apoptosis, survival, motility, invasion, and morphogenesis. Most miRNA genes are located in cancer-associated genomic regions (CAGRs) or fragile sites and act as oncogenes or tumor suppressor genes in the carcinogenic process of many cancers, including oral cancer. As the expression pattern of miRNAs changes under pathogenic conditions, the idea of using these stable molecules for clinical applications such as early diagnosis or prognosis has received a great deal of attention in recent years (Hydbring and Badalian-Very, 2013). A study on the expression levels of 72 miRNAs in oral cancer showed that miR-125a, miR-184, and miR-16 were up-regulated, while miR-69 was downregulated in oral tumor tissues compared with that in normal oral tissue. This study suggested these miRNAs as potential biomarkers for oral cancer (Santhi *et al*., 2013). We previously reported that miR7111-5p and miR6870-5p were downregulated in OSCC tissues, compared to adjacent tissues, which may be potential diagnostic and prognostic biomarkers for OSCC (Saraei *et al*., 2021). To our best knowledge, there is no study evaluating miR-589-5p and miR-569 expression in OSCC, while their aberrant expression has been frequently reported in various types of cancer. For instance, down-regulation of miR-589 was reported in cisplatin-resistant non-small cell lung cancer A549 cells (Li *et al*., 2016). A study by Liu *et al*. reported that miR-589-5p was drastically decreased in NSCLC compared with normal lung tissues. Furthermore, their founding showed a negative correlation between miR-589-5p and Histone deacetylase 5 (HDAC5) in NSCLC specimens. Given the regulatory role of the miR-589-5p/HDAC5 pathway in proliferation, migration, invasion, and tumorigenicity in NSCLC cells, this pathway was suggested as a new prognostic biomarker and therapeutic target against NSCLC (Liu *et al*., 2017). It was also found that the expression of miR-589-5p down-regulated in other cancers such as prostate (Ji *et al*., 2019) and triple-negative breast cancer (TNBC) (Chu, 2019). These results suggest that miR-589-5p through oncogenic or tumor-suppressive functions may be a potential biomarker in OSCC and different cancers. However, further research is needed to verify these findings.

A study by Zheng *et al*. showed that miR-569 expression levels were drastically decreased in human large-cell lung cancer (LCC). This work demonstrated that ectopic overexpression of miR-569 resulted from transfecting the cells with the miR-569 mimic induced cell apoptosis and prevented cell proliferation and migration. As reported in this study, miR-569 functioned as a potential tumor suppressor gene by downregulating HMGA2 and c-Fos expression levels in lung cancer (Zheng *et al*., 2013). The present study was carried out to explore novel diagnostic or prognostic biomarkers for OSCC. Consistent with these reports, our results demonstrated miR-589-5p and miR-569 highly down-expressed in OSCC tissues compared with the matched normal tissues.

c-Fos is a proto-oncogene that overexpressed in several cancers, such as cervical cancer (Cheung *et al*., 1997), lung cancers (Wodrich and Volm, 1993), pancreatic carcinomas (Lee and Charalambous, 1994), osteosarcomas (Wu *et al*., 1990), hepatocellular carcinoma (Arbuthnot *et al*., 1991) and colorectal adenoma (Magrisso *et al*., 1993). In oral cancer, *c-Fos* is reported to be serially overexpressed in 70% of squamous cell carcinoma of the oral cavity, 38% of premalignant lesions, and 16% in the normal oral mucosa (Sachdev *et al*., 2008). We applied bioinformatic analysis to uncover *c-Fos* as a direct target of miR-589-5p and miR-569. In the present study, we identified a significant increase in the expression level of the *c-Fos* gene in tumor tissues compared to adjacent normal tissues from OSCC patients, which

supports previously reported results. In contrast to our findings, De Sousa *et al*. have demonstrated that *c-Fos* down-regulated in OSCC tissues compared to the normal oral mucosa (De Sousa *et al*., 2009). Similarly, another study indicated that the expression level of *c-Fos* mRNA and protein was significantly lower in oral carcinoma compared with normal tissue of the oral cavity. In addition, they found that overexpression of *c-Fos* was observed in low-grade tumors with early stages (Krishna *et al*., 2018). In the present study, we correlated the expression level of miR-589-5p and miR-569 to *c-Fos* mRNA and found inverse correlations between miR-589-5p (r=−0.0526, p= 0.7825), miR-569 (r=−0.1026, p= 0.5897).

Muhammad *et al.* demonstrated that overexpression of *c-Fos* enhanced tumor growth in xenograft mouse models of HNSCC (Muhammad *et al*., 2017). We have observed *c-Fos* overexpression in late-stage tumors *(*grade II) than in early-stage tumors (grade I) and found no significant correlation (*p*=0.455). However, in contrast, in the study by Krishna *et al*. higher proportion of low-grade tumors with early stages were more prone to overexpression of c-Fos (Krishna *et al*., 2018). However, we did not find a significant relation between *c-Fos* expression and tumor size in OSCC samples, although the expression of *c-Fos* was slightly higher in larger tumors (2-5 cm and >5 cm) than in small tumors (<2 cm) (*p*=0.895). The association between c-Fos expression and lymph node metastasis in oral cancer has been addressed in some studies. Wang *et al.* found a significant association between c-jun or c-Fos expression with lymph node metastasis. They showed that co-expression of c-jun/c-Fos or c-jun/c-Fos/p53 was significantly associated with lymph node metastasis and poor differentiation in OSCC cases (Wang *et al*., 2016). In another study, *c-Fos* overexpression was found in OSCC patients with lymph node involvement, compared to patients without that (Krishna *et al*., 2018). In contrast with these findings, we found no significant relation between *c-Fos* overexpression and lymphatic involvement (*p*=0.895). In addition, no remarkable association was found between vascular, perineural, and depth invasions with *c-Fos* overexpression in the present study. However, these findings may be at least partly due to the small sample size of this study, which is worthy of further investigation. Tumor necrosis is a frequent pathological event in most solid tumors, such as HNSCC (Li *et al*., 2020). The role of necrosis in cancer progression (Karsch-Bluman *et al*., 2019) and promotion of tumor microenvironment (Lotfi *et al*., 2016) has been determined. A significant association was found between *c-Fos* expression and necrosis status in OSCC tumors in the present study (*p*=0.034). Moreover, ROC curve analysis showed valid diagnostic values for miR-589-5p (0.865 (95% CI: 0.769–0.961; *p* <0.0001) that can be considered as novel diagnostic biomarkers for OSCC.

Further studies with larger sample size and in the context of c-Fos protein expression are required to confirm these findings and better understand the association between the *c-Fos* gene and the expression of miR-589-5p and miR-569 in the malignancy of oral cancer and alter the aggressive behavior of oral cancer cells, particularly in clinical studies.

Conclusion

The outcomes of this study uncovered the first evidence of evaluation of miR-589-5p and miR-569 expression in OSCC and demonstrated increased expression of the *c-Fos* gene and decreased expression of miR-589-5p and miR-569 in tumor tissues, compared to normal adjacent tissues of OSCC patients. miR-589-5p may serve as a potent diagnostic and prognostic biomarkers for

OSCC patients and be a potential approach to early diagnosis or prognosis of OSCC in the future.

Disclosure Statement: The authors reported no potential conflict of interest.

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