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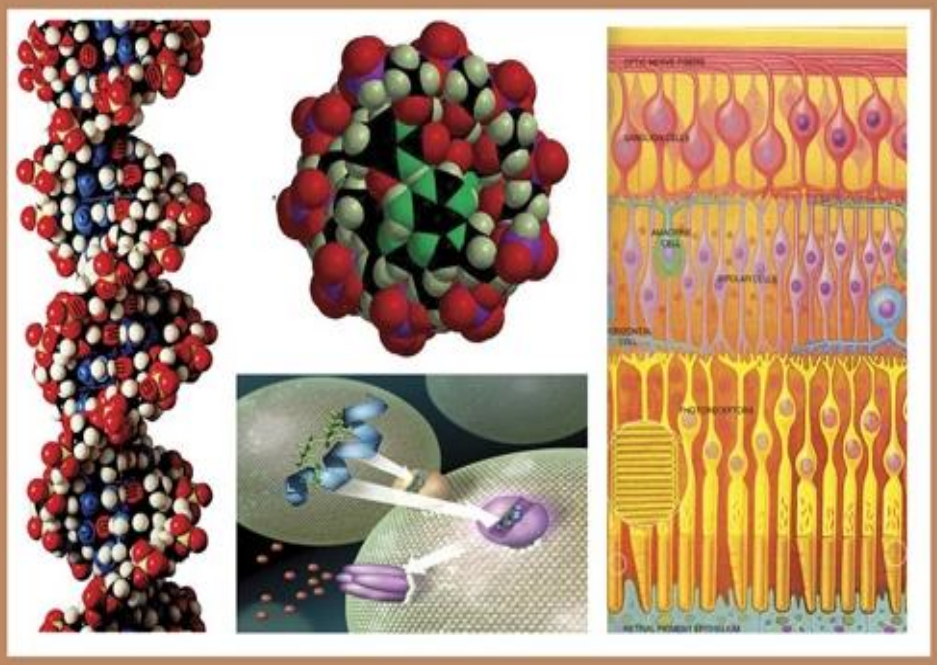

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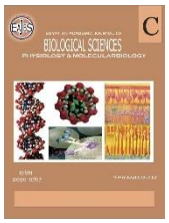

Diagnostic and Prognostic Value of miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 in Malignant Transformation of Oral Lichen Planus and OSCC

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ABSTRACT

Background/aims: Oral squamous cell carcinoma (OSCC) is the most common type of malignant neoplasm of the oral cavity and may become malignant from precancerous lesions such as oral lichen planus (OLP). This study aimed to determine the expression profile of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 to identify novel biomarkers for OSCC screening and prognostic predictors for the pre-malignant potential of OLP. **Methods:** The expression levels of these miRNAs, circRNAs, and *GRB2* target-gene in 30 OSCC and 10 OLP tissues compared to their adjacent-normal tissue samples were analyzed by qRT-PCR and compared clinicopathological characteristics in patients with OSCC. The potential diagnostic values of miRNAs and circRNAs in OSCC as risk factors of carcinogenesis were assessed by ROC curve analysis. **Results:** The expression levels of studied miRNAs and circRNAs were significantly lower in OSCC patients than in healthy controls ($p < 0.001$). Also, the expression of circ_0045638 and circ_0045639 were found significantly downregulated in OLP samples compared with healthy tissues but the expressions of miR-4789-5p, and miR-3941 were not significant ($p = 0.078$ and 0.074, respectively). Significant upregulation of the *GRB2* gene was found in OSCC patients relative to adjacent-normal tissues ($p < 0.001$). We found a negative correlation between the expression miR-4789-5p, circ_0045638, and circ_0045639 and *GRB2* in OSCC. Significant correlations were indicated between decreased expression of miR-4789-5p and perineural invasion, necrosis, and metastasis. The higher expression level of *GRB2* was correlated with tumor size and lymphatic invasion. Area under the ROC curve (AUC) of miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 were 0.971, 0. 821, 0.967 and 0.982, respectively, indicated their potential diagnostic value with excellent accuracy and specificity. **Conclusion:** miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 expression status may provide the possibility to establish an accurate screening method for OLP that is a crucial step in the prevention of malignant transformation.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm and one of the most common types of oral cancer (OC), accounting for more than 90% of all oral cavity cancers. It has become a global public health alert, with approximately 350,000 new cases diagnosed and more than 177,000 deaths annually worldwide (Pires *et al*., 2013; Coletta *et al*., 2020).

OSCC is characterized by local invasiveness and early cervical lymph node metastasis, diagnosed in the late stage of pathogenic tumor node metastasis pTNM (III-IV) (Rakia *et al*., 2018). Despite the recent breakthroughs in therapy, OSCC-related mortality has remained steadily high due to poor prognosis, late diagnosis, and recurrence (Wang *et al*., 2018). Thus, a better understanding of the OSCC molecular pathogenesis is essential to develop novel approaches for the timely and accurate diagnosis of OSCC, resulting in increased patients' chances of survival and quality of life. microRNAs (miRNAs) and circular RNAs (circRNAs) are the most studied classes of non-coding RNAs (ncRNAs) that play significant roles in almost all of the hallmarks of cancer, especially tumorigenesis and metastasis. A growing number of studies have shown that altered regulations miRNAs and circRNAs as either oncogene (miR-21, miR-146a, miR-155, circ_0002185, circ_0001821) or tumor suppressors (miR-320, miR-7, miR-218, circ_0002203, circ_0004491) are closely associated with OSCC initiation, progression, and metastasis (Anjie *et al*., 2015; Wang *et al*., 2018; Chamorro Petronacci *et al*., 2019). miRNAs are small single-stranded sequences of (19–24 nucleotides) RNA transcripts that exert changes in the post-transcriptional regulation of target mRNAs through binding to their complementary sequence of 3′untranslated region (UTR), resulting in degradation or the suppression of mRNA translation (He *et al*., 2019; Noraini *et al*., 2020). circRNAs are novel single-stranded closed circular non-protein coding RNA without 3′-poly (A) tails and 5′-caps (Cheng *et al*., 2021). Although circRNAs can be involved in the regulation of tumorigenesis through many functions, including protein antagonists, interacting with regulatory RBPs (RNA-binding proteins), transportation, and communication, however, mainly serve as miRNA sponges by forming a regulatory axis with microRNA (Xiaozhu *et al*., 2021). Oral lichen planus (OLP) is a chronic autoimmune disease of the oral mucosa and has been considered a potentially malignant disorder since the first WHO workshop in 2005. Although OLP is known to be associated with a low rate of malignant transformation ranging from 0-9%, long-term monitoring of patients with OLP may lead to a decrease in the rate of malignant potential of OLP (Radochová *et al*., 2021). Overexpression of *GRB2* (growth factor receptor-bound protein 2) is related to cancer hallmarks such as cell proliferation, invasion, and metastasis through the Ras/Raf/MEK/mitogen-activated protein (MAP) kinase pathway. Several studies have indicated that direct and indirect interactions of Grb2 with various signaling molecules have been involved in the onset and progress of multiple human cancers, including breast, bladder, gastric, OSCC, and colorectal cancers (Giubellino *et al*., 2008; Li *et al*., 2014).

In the present study, we selected miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 from bioinformatics databases (CircInteractome, TargetScan, Mirwalk, and miRDB), and *GRB2* was predicted as the targeted gene. Although expression patterns of several miRNAs and circRNAs to achieve promising diagnostic biomarkers in OSCC have been frequently studied, no studies have been performed on the role of miR-4789-5p miR-3941, circ_0045638, and circ_0045639 in OSCC. Therefore, we aimed to identify the differential expression of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 in OSCC and OLP samples compared with healthy-adjacent tissue.

MATERIALS AND METHODS

Patients and Samples:

All protocols of the present study were approved by the Research Medical Ethics Committee of Imam Khomeini Hospital (IR.IAU.SRB.REC.1399.099). All patients signed written informed consent to use clinical specimens for research purposes. This study included samples from 40

individuals categorized into four groups: i) OSCC tissue samples (n = 30); ii) adjacent normal oral epithelial tissues obtained from the OSCC patients as control for OSCC (n = 30); iii) OLP tissue samples (n= 10) and iv) matched adjacent normal tissue samples as control for OLP (n = 10). OSCC samples were collected from patients before any therapeutic procedures by the Iran National Tumor Bank, Cancer Institute, Imam Khomeini Hospital, Tehran, from October 2020 to January 2021. The OSCC and OLP were diagnosed by histopathological examination according to the diagnostic criteria of the World Health Organization (Pindborg *et al*., 1997). The tissues were obtained from patients under the supervision and approval, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Clinicopathological information such as age, gender, tumor stage, and other variables was collected from patients' medical records. The degree of tumor differentiation was classified into well, moderately, and poorly differentiated squamous cell carcinoma, according to World Health Organization (Pindborg *et al*., 1997).

miRNAs and circRNAs Target Prediction:

The Circinteractome database (https://circinteractome.irp.nia.nih.gov/), TargetScan (http://www.targetscan.org), Mirwalk (http://mirwalk.uni-hd.de), and miRDB (http://mirdb.org) databases were used to predict potential miRNA target genes. The selected gene for both miRNAs was subjected to analysis.

RNA Extraction, Reverse Transcription and Quantitative Real Time-PCR (qRT-PCR):

Extraction of total RNA from samples was performed using the TRIZOL reagent (Invitrogen, Sigma, USA), according to the manufacturer's protocol and the amount quantified using a Nanodrop spectrophotometer (Thermo scientific-Nanodrop 2000). Complementary DNA (cDNA) was synthesized using the BioFACT cDNA Synthesis kit (Daejeon, South Korea). The appropriate stem-loop RT primers have been used to synthesize cDNA for miRNAs using the MiR-Amp kit (Parsgenome, Iran). Total RNA was incubated with RNase R for 15 min at 37 °C to deplete the linear RNAs and reverse transcribed to produce circRNAs cDNA with random hexamers using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA), following the manufacturer's guidelines. The level of miR-4789-5p, miR-3941, circ_0045638, circ_0045639, and *GRB2* were analyzed on a LightCycler TM 96 (Roche) using SYBR Green Master Mix (TAKARA, Japan). The cycling programs were as follows: 95°C for 5 min and 40 cycles comprising 95°C for 15sec, 60°C for 30 sec and 72°C for 20 sec. ACTB was used to normalize the expression of *GRB2*, while U6 was applied as a housekeeping gene for miRNAs and circRNAs. The expression levels were measured using the $2^{-\Delta\Delta Ct}$ method (Li *et al*., 2014). All the primers were designed using Primer3plus software, and sequences have been shown in Table 1.

Table 1. primer sequences used for RT-qPCR

Target transcript	Primer type	Sequence (5′→3′)
circ_0045638	Forward	TTTGGAAACGATGTGCAGCA
	Reverse	TGGCACCTGTTCTATGTCCC
circ_0045639	Forward	AGACGGCTTCATTCCCAAGA
	Reverse	TGCTGCACATCGTTTCCAAA
miR-4789-5p	Forward	GATACACCAGATAGAGATAG
	Reverse	GAACATGTCTGCGTATCTC
miR-3941	Forward	TTACACACAACTGAGGATCA
	Reverse	GAACATGTCTGCGTATCTC
GRB2	Forward	GACGAGCTGAGCTTCAAAAGG
	Reverse	CGTTTCCAAACTTGACAGAGAGG
ACTB	Forward	GATCAAGATCATTGCTCCTCCTG
	Reverse	CTAGAAGCATTTGCGGTGGAC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AGAGCAGGGTCCGAGGT

Statistical Analysis:

All experiments were repeated three times, and numerical data were expressed as the means ± standard deviation. Statistical analysis was performed using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS (GraphPad Prism 5 software (version 18.0; SPSS, Inc., Chicago, IL, USA). Expression data were controlled for normal distribution by one-sample Kolmogorov-Smirnov (K–S test). A one-way ANOVA was used to determine statistical differences in *GRB2* gene expression levels. The associations between miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 and *GRB2* levels and clinic-pathological parameters of OSCC patients were assessed using an independent sample test and independent-sample Kruskal-Wallis test. The correlation between miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 and *GRB2* expression was performed by Pearson correlation analysis. 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-4789-5p and miR-3941 Expressions Were Downregulated in OSCC and OLP Tissues:

qRT-PCR was performed to examine miR-4789-5p and miR-3941 expression levels in OSCC (n=30) and OLP tissues (n=10) compared to their adjacent-normal tissue samples. As shown in Figures 1a and c, miR-4789-5p and miR-3941 were markedly downregulated in OSCC samples compared to adjacent normal samples (P <0.0001). In addition, we found that miR-4789-5p and miR-3941 expression levels were slightly decreased in OLP compared to adjacent normal tissue and were not considered meaningful according to the statistical analysis (P > 0.05, Figs. 1b and d). The expression of miR-4789-5p and miR-3941 was decreased in OSCC patients compared with OLP patients but was not statistically significant (p = 0.078 and 0.074, respectively). The fold-change for miR-4789-5p was 4.03-fold, whereas this value amounted to 7.3 for miR-3941.

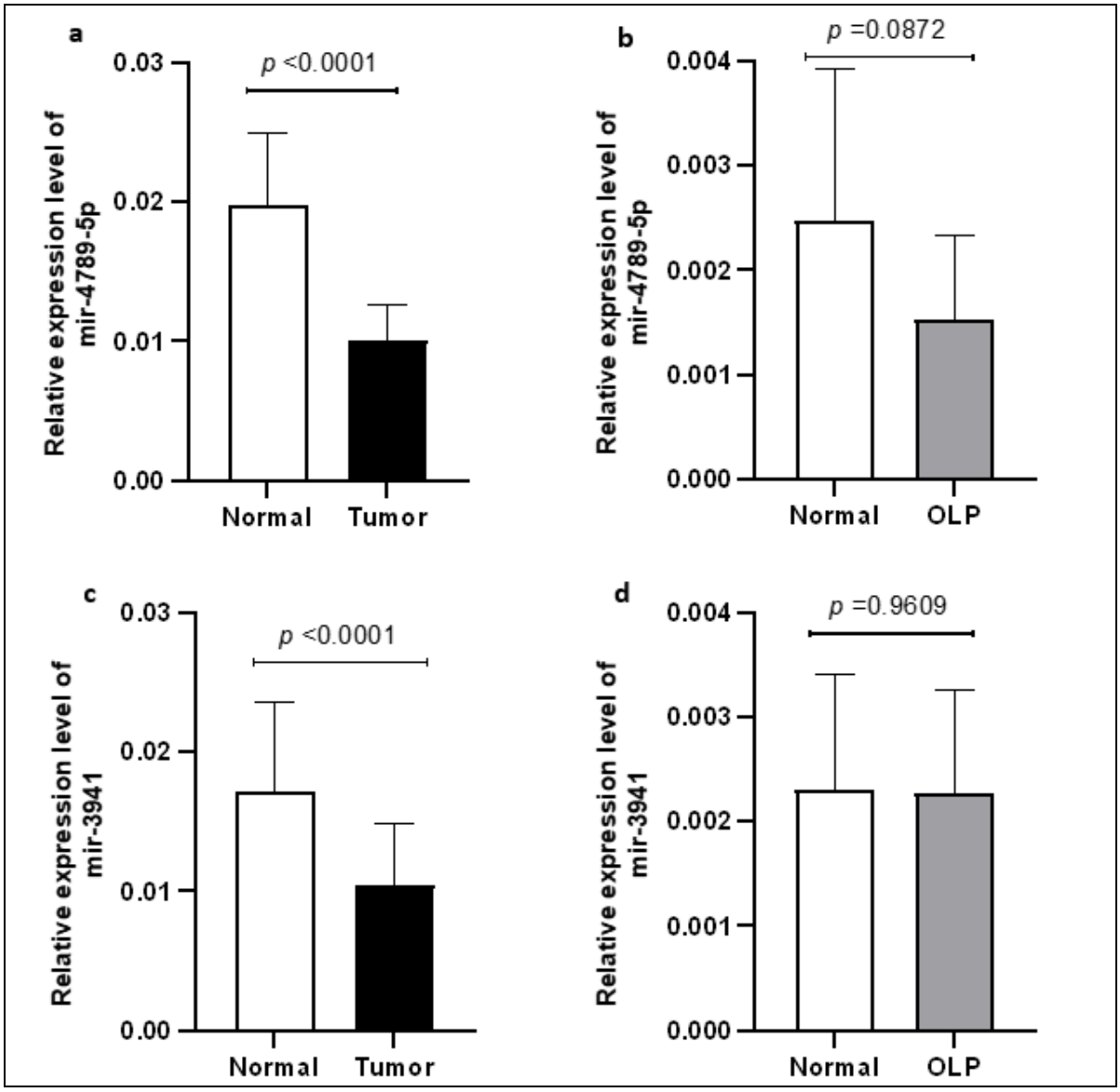

Fig.1: Quantitative RT-PCR analysis of miR-4789-5p and miR-3941, circ_0045638 expressions in OSCC tissues and adjacent normal tissues (n=30) (a and c), OLP (Oral lichen planus), and adjacent normal tissues (n=10) (b and d). Transcript levels were normalized to U6 expression. Data are presented as means ± SD.

circ_0045638 and circ_0045639 Expressions Were Downregulated in OSCC and OLP Tissues:

We also assessed the expression levels of circ_0045638 and circ_0045639 in 30 OSCC patients and 10 OLP patients compared to their adjacent-normal tissue samples. circ_0045638 and circ_0045639 were significantly downregulated in OSCC and OLP samples compared to normal tissues (P <0.0001, Figs. 2a-c). The levels of circ_0045638 and circ_0045639 were decreased by 1.47 and 2.02-fold in OSCC patients than in OLP; however, this difference was not statistically significant (p = 0.40 and 0.143, respectively).

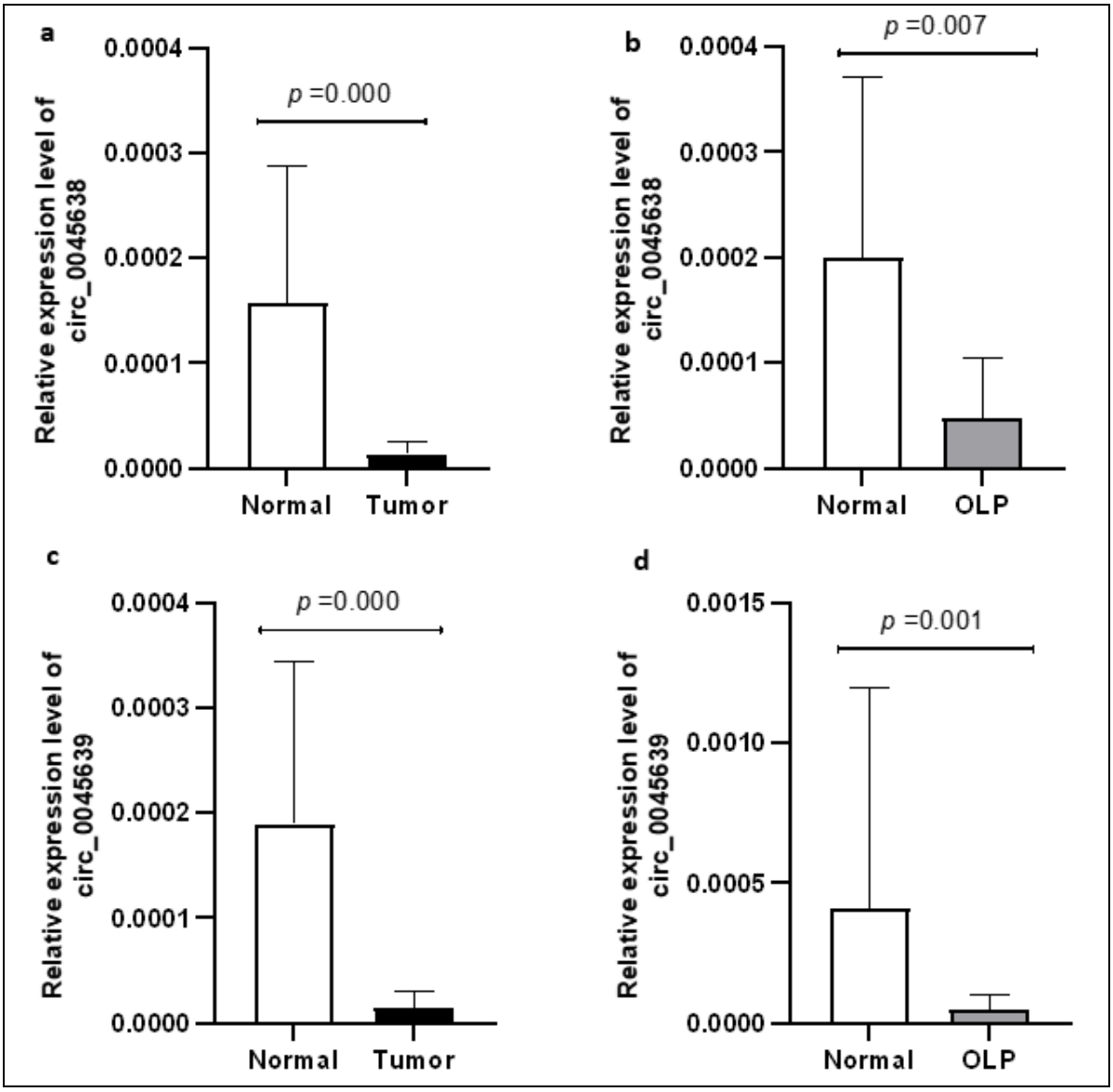

Fig. 2 Quantitative RT-PCR analysis of circ_0045638 and circ_0045639 expressions in OSCC tissues and adjacent normal tissues (n=30) (a and c), OLP (Oral lichen planus), and adjacent normal tissues (n=10) (b and d). Transcript levels were normalized to U6 expression. Data are presented as means ± SD.

***GRB2* Gene Expression Was Upregulated in OSCC and OLP Tissues:** We used CircInteractome, TargetScan, Mirwalk, and miRDB algorithms to uncover the putative co-targets gene of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 in OSCC. Based on the bioinformatics prediction analysis, *GRB2* was selected as a potential direct target for corresponding miRNAs and circRNAs. As a result, *GRB2* expression levels were up-regulated in OSCC tissue samples relative to adjacent-normal tissue samples (p >0.0001; Figure 3a). Regarding OLP cases, the relative expression level of *GRB2* was slightly higher than that in adjacent normal tissues but insignificant (p =0.9546; Fig. 3b). In addition, the expression of *GRB2* was downregulated 3.02-fold in OSCC patients than in OLP; however, this difference was not statistically significant (p = 0.38).

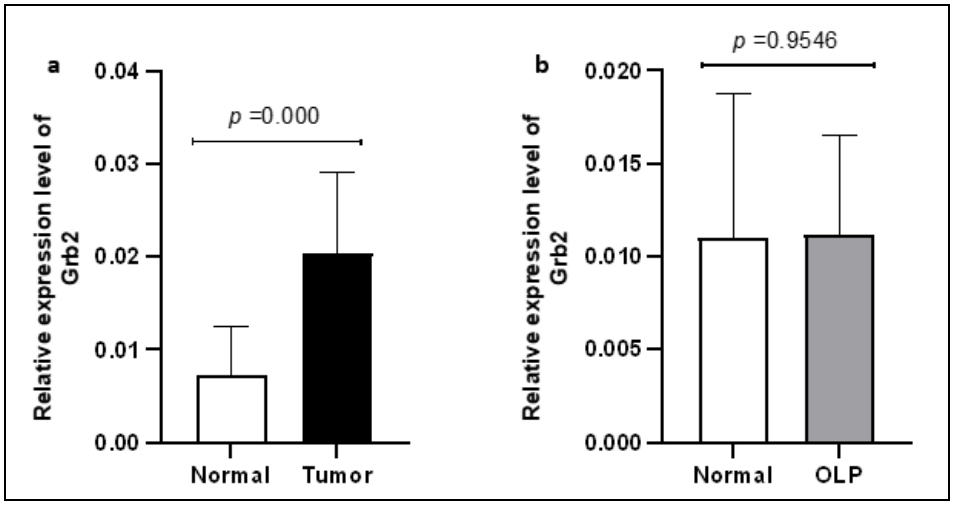

Fig. 3 Quantitative RT-PCR analysis of *Grb2* expression in OSCC tissues and adjacent normal tissues (n=30) (a), OLP (Oral lichen planus), and adjacent normal tissues (n=10) (b). Transcript levels were normalized to ACTB expression. Data are presented as means ± SD.

Correlation between *GRB2* Expression and miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 in OSCC Patients:

Pearson's correlation analysis was performed to determine whether the expression of *GRB2* was associated with miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 in OSCC. We observed an inverse correlation between miR-4789-5p, circ_0045638, and circ_0045639 in OSCC with overexpression of *GRB2* but differences were not significant (r=−0.2869, p= 0.1243; r=−0.1017, p= 0.5927; and r=−0.0930, p= 0.6247, Figs. 4a, c and d). miR-3941expression was not significantly correlated with the expression of overexpression of *GRB2* target gene (r=0.0012, p=0.9950; Fig. 4b).

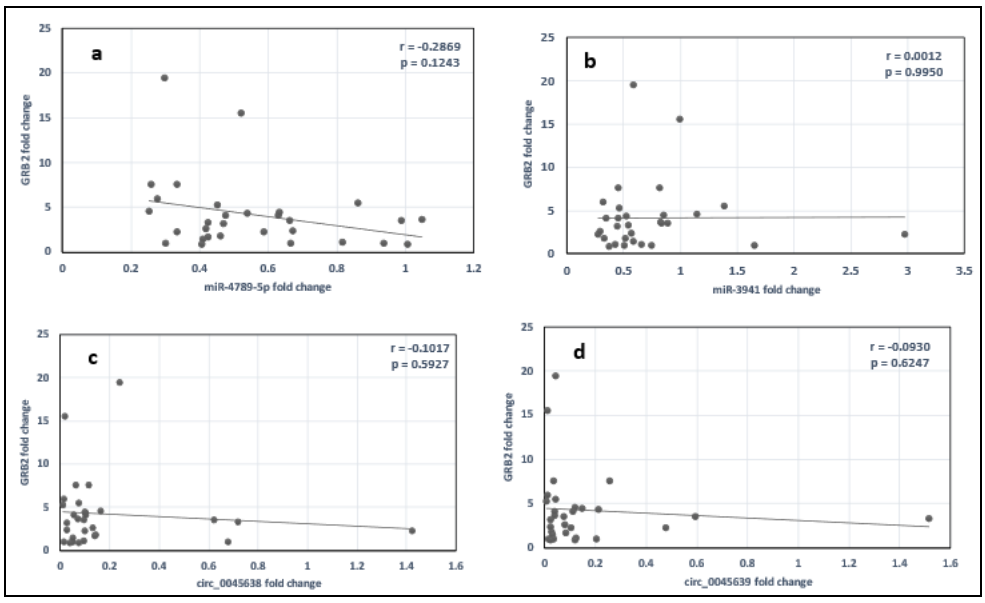

Fig. 4 Pearson's correlation analysis between GRB2 mRNA expression and miR-4789-5p (a), miR-3941 (b), circ_0045638 (c) and circ_0045639 (d) levels in OSCC patients. Data are presented as means ± SE. No significant correlation was found between the expression of *GRB2*, miRNAs, and circRNAs in OSCC patients' tissues. **P* < 0.05.

Association between miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 Expression Levels and Clinicopathological Features in OSCC:

We next examined the associations of miR-4789-5p, miR-3941, circ_0045638, circ_0045639 and *GRB2* target gene with the clinicopathological features of patients with OSCC as summarized in **Table 2.** The results revealed that the level of the miR-4789-5p expression in OSCC was significantly associated with perineural invasion (p = 0.026), necrosis (p = 0.007) and metastasis (p = 0.048). The low expression level of miR-3941 was significantly associated with the tumor stage (p = 0.006). We found no significant relationship between these two miRNAs expression and other clinicopathological characteristics of OSCC (p>0.05). As shown in Table 2, gender (p = 0.030), age (p = 0.01), tumor size (p = 0.031), grade (p = 0.006) and metastasis (p= 0.000) were related with low circ_0045638 expression. circ_0045639 expression was positively correlated with perineural, vascular and lymphatic invasions (p = 0.022, 0.002 and 0.000, respectively) as well as necrosis (p = 0.008) but was not associated with other clinicopathological features (p> 0.05). In addition, *GRB2* expression showed a significant correlation with tumor size (p = 0.001) and lymphatic invasion (p = 0.045) but was not associated with other clinicopathological features other clinicopathological variables, including age, gender, tumor stage and metastasis, etc.(p> 0.05).

Table 2: Association of miR-4789-5p, miR-3941, circ_0045638, circ_0045639 and GRB2 target gene expression with clinicopathological characteristics in OSCC.

Clinical features	Case No. (%)	miR-4789-5p		miR-3941		circ_0045638		circ_0045639		*GRB2*	
		Mean±SD	*P* value	Mean±SD	*P* value	Mean±SD	*P* value	Mean±SD	*P* value	Mean±SD	*P* value
Gender			0.370		0.234		**0.030**		0.105		0.145
Female	8	0.56±0.28		0.62±0.25		0.06±0.03		0.05±0.03		2.44±1.37	
Male	22	0.54±0.22		0.76±0.60		0.22±0.34		0.18±0.33		4.87±4.58	
Age (Years)			0.437		0.198		**0.001**		0.984		0.476
≥ 40	26	0.55±0.22		0.76±0.56		0.14±0.200		0.15±0.30		4.40±4.34	
< 40	4	0.5266±0.32		0.44±0.10		0.41±0.67		0.16±0.20		3.11±1.96	
Size(cm)			0.061		0.788		**0.031**		0.138		**0.001**
≥ 5	9	0.52±0.17		0.69±0.41		0.07±0.02		0.06±0.07		5.77±6.92	
< 5	21	0.56±0.25		0.73±0.58		0.23±0.34		0.19±0.33		3.56±1.93	
Perineural invasion			**0.026**		0.427		0.437		**0.022**		0.705
Present	12	0.53±0.17		0.80±0.71		0.24±0.26		0.24±0.43		3.75±5.10	
Absent	18	0.56±0.27		0.66±0.38		0.14±0.32		0.09±0.11		4.54±3.41	
Vascular invasion			0.990		0.887		0.192		**0.002**		0.251
Present	6	0.69±0.22		0.76±0.46		0.26±0.33		0.33±0.58		3.38±1.70	
Absent	24	0.51±0.22		0.71±0.55		0.16±0.29		0.10±0.14		4.44 ±4.51	
Lymphatic invasion			0.790		0.937		0.789		**0.000**		**0.045**
Present	5	0.71±0.23		0.93±0.44		0.21±0.29		0.36±0.65		7.28±6.91	
Absent	25	0.51±0.22		0.68±0.54		0.17±0.30		0.10±0.14		3.61±3.17	
Necrosis Presence			**0.007**		0.736		0.750		**0.008**		0.214
Present	7	0.61±0.33		0.66±0.38		0.16±0.24		0.26±0.55		5.16±4.76	
Absent	23	0.53±0.20		0.74±0.57		0.19±0.31		0.11±0.14		3.94±3.96	
Stage			0.233		**0.006**		0.057		0.205		0.756
Low (I and II)	13	0.52±0.27		0.97±0.72		0.13±0.15		0.10±0.15		4.62±4.60	
High (III and IV)	17	0.57±0.20		0.52±0.16		0.22±0.37		0.19±0.36		3.92±3.80	
Grade			0.659		0.441		**0.006**		0.083		0.996
I	16	0.52±0.23		0.85±0.62		0.12±0.14		0.09±0.14		4.19±4.45	
II	13	0.59±0.24		0.57±0.38		0.26±0.42		0.23±0.40		4.52±3.87	
Status unknown	1										
Clinical Metastasis			**0.048**		0.684		**0.000**		0.265		0.639
M0	29	0.53±0.22		0.73±0.54		0.14±19		0.14±0.28		4.29±4.16	
M1	1	1.00		0.50		0.14		0.47		2.27	

Potential Diagnostic Values of miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 in OSCC:

To evaluate whether the tissue expression levels of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 could be used as predictive factors of carcinogenesis in OSCC, we examined the potential diagnostic values of these molecules by ROC curve analysis. The ROC curves for miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 were plotted and values of the area under the curve (AUC) were 0.971 (95% CI: 0.938–1.000; p <0.001) , 0.821 (95% CI: 0.712–0.930; p <0.001), 0.967 (95% CI: 0.931–

1.000; p <0.001) and 0.982 (95% CI: 0.955–1.000; p <0.001), respectively (Figs. 5a-d). The expression level of circ_0045639 showed higher discriminatory accuracy between the OSCC and healthy tissues relative to others, with an AUC of 0.982 (95% CI: 0.955–1.000; Fig. 5a). The ROC curve analysis indicated that the expression levels of the above miRNAs and circRNAs could serve as potential biomarkers for distinguishing patients with OSCC from healthy controls.

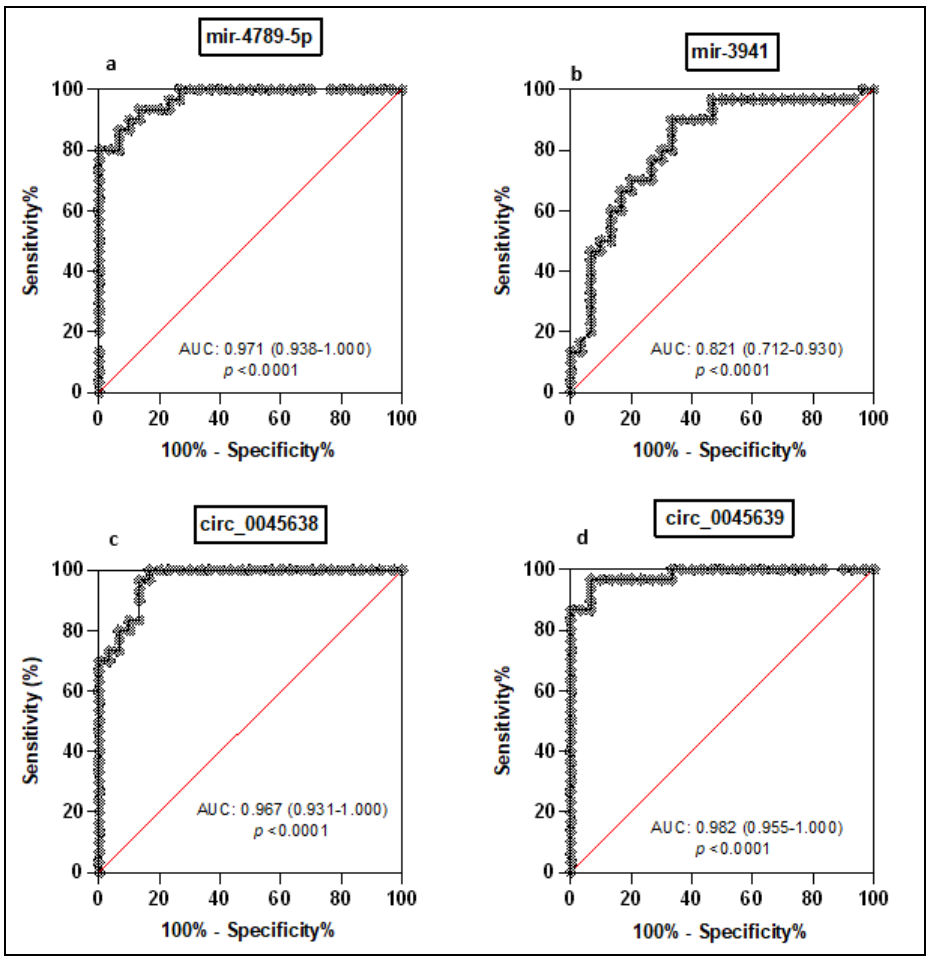

Fig 5. ROC curve analyses related to the expression of miR-4789-5p (a), miR-3941 (b), circ_0045638, (c) and circ_0045639 (d) to discriminate patients with OSCC from healthy controls.

DISCUSSION

OSCC is one of the most prevalent cancers which causes cancer-related deaths worldwide due to late diagnosis in advanced stages (III and IV) (Jain *et al*., 2021). Therefore, identifying reliable biomarkers to serve as accurate indicators is essential to early diagnosis and new targeted therapies. Numerous ongoing research efforts are focused on identifying methods based on ncRNAs, such as miRNAs and circRNAs for cancer diagnosis and prognosis due to their remarkable proven roles in carcinogenesis, metastasis, recurrence, tissue-specific expression, the non-invasive and convenient nature of diagnostic procedures (Markou *et al*., 2015; Wang *et al*., 2021). Recent studies have detected aberrant microRNA expression patterns in OSCC using microarray, qRT-PCR, Northern blotting, and deep sequencing-based approach, which have led to the identification of many oncogenic and tumor suppressor miRNAs (Xiqiang *et al*., 2009; Wang *et al*., 2018). Up to now, a growing number of miRNAs and circRNAs have been reported to be dysregulated in OSCC.

A study by Tesi *et al*. (Tsai *et al*., 2017) reported that miR-375 and miR-204 were down-regulated, while miR-196a was highly expressed in 39 pairs of OSCC samples. Shi *et al*. (Shi *et al*., 2019) suggested that low expression of miR-106a could not suppress OSCC cell proliferation and EMT through direct downregulation of LIMK1 expression. Our previous study revealed that miR7111-5p and miR6870-5p were significantly downregulated in OSCC tissue relative to adjacent normal oral tissues (Sarae *et al*., 2021). Similarly, the results of two studies showed that miR-433 expression was significantly reduced in OSCC and indicated that its overexpression markedly suppressed the proliferation, invasion, and migration of OSCC through sponge function of Linc01234 and targeting HDAC6 (Wang *et al*., 2015; Liu *et al*., 2020). Wang *et al*. (Wang *et al*., 2018) found eight up-regulated and down-regulated circRNA molecules in eight pairs of OSCC patients by high-throughput Sequencing. Among these, the reduced expression of circ_000334, circ_006740, and circ_006371 related to pathological differentiation in 42 pairs of OSCC samples was confirmed by qRT-PCR. Ai *et al*. (Ai *et al*., 2020) reported the sponge function of circRNA, where the overexpression of circ_SEPT9 dramatically promoted OSCC cell proliferation and metastasis through direct downregulation of miR-1225 expression. hsa_circ_0003829 and hsa_circRNA_100533 were down-expressed in OSCC tissues while their overexpression was shown suppressed proliferation, migration, and extended cell apoptosis in OSCC (Zhu *et al*., 2019; Zhang *et al*., 2020).

Although dysregulation of miRNAs and circRNAs molecules is suggested their potential as promising diagnostic biomarkers in the clinical monitoring of OSCC, further studies are needed to establish specific miRNAs signatures in predicting and diagnosing OSCC. Therefore, in the present study, we provided the first investigation of the diagnostic and prognostic value of miR-4789-5p and miR-3941 and circ_0045638 and circ_0045639 expressions in OSCC. We discovered that the expression of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 were significantly downregulated in 30 healthy and tumor tissue pairs of OSCC patients. Leukoplakia, OLP, and erythroplakia are the most common potential precancerous oral lesions that have a high potential for malignant transformation (Maia *et al*., 2016). Regardless of visual inspection of ulceration status, it is very relevant to identify biomarkers to support the early detection of malignant neoplasms from pre-malignant or primary lesions and could be a successful step toward timely diagnosis and treatment. To the best of our knowledge, no previous study has evaluated the expression of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 in precancerous lesions and OSCC. With this motivation, we also assessed the expression levels of these miRNAs and circRNAs in 10 pairs of samples of OLP. We demonstrated that levels of these miRNAs and circRNAs were also reduced in OLP relative to their matched-adjacent normal tissues. We found that while the expression levels of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 were diminished in OSCC than in OLP, this alteration was not significant according to the statistical analysis (p = 0.078, 0.074, 0.400, and 0.143, respectively). These findings suggest that alteration in the expression pattern of these miRNAs and circRNAs may serve as effective biomarkers for early diagnosis of OSCC and timely prevent malignant OLP transformation through monitoring patients. We applied bioinformatic analysis to uncover *GRB2* as a direct target of miR-4789-5p, miR-3941, circ_0045638, and circ_0045639. In our study, *GRB2* expression was significantly up-regulated in OSCC (p =0.000) while slightly elevated in OLP compared with corresponding non-tumor tissues (p = 0.954). In the present study, we also correlated the expression level of miRNAs and circRNAs

to GRB2 mRNA. We found inverse correlations between miR-4789-5p (r = − 0.2869), circ_0045638 (r = − 0.1017), circ_0045639 (r = − 0.0930), and *GRB2* that suggesting their negative regulation on *GRB2*. Li *et al.* reported that *GRB2* was overexpressed in OSCC tissues and significantly correlated with lymph node metastasis (Li *et al*., 2014). In agreement with that, we also found significant correlations between *GRB2* overexpression and lymphatic invasion (p = 0.045) and tumor size (p = 0.001) in OSCC patients. Lymphatic invasion is one of the predictors of lymph node metastasis (Fujimoto *et al*., 2007). Therefore, it is suggested that *GRB2* overexpression is associated with OSCC progression and may serve as a prognostic factor to predict the existence of regional lymph node metastasis. Numerous studies have revealed that abnormal expression of miRNAs and circRNAs are closely associated with clinicopathological features (tumor size, lymph node metastasis, TNM stage, pathological differentiation, etc.) and survival outcomes of OSCC patients (Sun *et al*., 2018; Li *et al*., 2020; Peng *et al*., 2020; Wei *et al*., 2020; Tao *et al*., 2021). In the current study, the mir-4789-5p expression level was significantly associated with clinicopathological characteristics of OSCC patients, including perineural invasion, necrosis, and clinical metastasis. Furthermore, the expression level of circ_0045638 was significantly associated with tumor grade and metastasis while circ_0045639 with invasions and necrosis. These findings suggest that circ_0045638 and circ_0045638 down expression is associated with carcinogenesis and progression of OSCC and may serve as potential diagnostic biomarkers for OSCC. Moreover, ROC curve analysis showed valid diagnostic values for all miR-4789-5p, miR-3941, circ_0045638 and circ_0045639 (0.971 (p <0.001), 0. 821 (p <0.001), 0.967 (p <0.001) and 0.982 (p <0.001), respectively) that can thus be considered as novel diagnostic biomarkers for prognosis and screening of OSCC.

Conclusion

In conclusion, these results provided the first evidence that miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 expression were significantly downregulated in OSCC patients and may serve as promising diagnostic and prognostic biomarkers for OSCC patients. miR-4789-5p, miR-3941, circ_0045638, and circ_0045639 expression status may provide the possibility to establish an accurate screening method for OLP that is a crucial step in the prevention of malignant transformation. Further studies with large samples could reflect and confirm more clarifying results.

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