MiR-638 suppresses the progression of oral squamous cell carcinoma through wnt/β-catenin pathway by targeting phospholipase D1

Kai-Liang Tang\textsuperscript{a,b}, Han-Ying Tang\textsuperscript{c}, Yi-Du\textsuperscript{b}, Tian Tian\textsuperscript{d} and Shi-Jiang Xiong\textsuperscript{a}

\textsuperscript{a}Department of VIP Center and Shandong Provincial Key Laboratory of Oral Biomedicine, School and Hospital of Stomatolagy, Shandong University, Jinan, Shandong, China; \textsuperscript{b}Department of Endodontics, Jinan Stomatological Hospital, Jinan, Shandong, China; \textsuperscript{c}Department of Oral prostholgy, Jinan Stomatological Hospital, Jinan, Shandong, China; \textsuperscript{d}Department of Stomatolagy, Affiliated Hospital of Binzhou Medical College, Binzhou, Shandong, China

ABSTRACT

Objective: The current study aimed to explore the function of miR-638 on the progression of oral squamous cell carcinoma (OSCC) and relevant molecular mechanisms.

Methods: Expression profile of miR-638 in OSCC tissues and cells was detected using quantitative real-time polymerase chain reaction (qRT-PCR) method. Chi-square test was performed to estimate the relationship between miR-638 and clinical parameters of OSCC cases. Cell viability and motility abilities were estimated using MTT and transwell assays, respectively. Potential targets of miR-638 in OSCC were identified through bioinformatics analysis and luciferase reporter assay.

Results: MiR-638 exhibited decreased expression in OSCC tissues and cells, compared to non-cancerous controls (P < .05 for both). Moreover, its down-regulation was closely correlated with lymph node metastasis (P = .044) and TNM stages (P = .001). Enforced miR-638 expression reduced cell proliferation, migration and invasion, while its knockdown exhibited opposite effects. Phospholipase D1 (PLD1) was confirmed as a target of miR-638 in OSCC. MiR-638 could inhibit wnt/β-catenin pathway through targeting PLD1, thus realizing its anti-tumour action in OSCC.

Conclusion: MiR-638 may be a tumour suppressor in OSCC by targeting PLD1/Wnt/β-catenin pathway.

Introduction

Oral squamous cell carcinoma (OSCC) is a frequently diagnosed malignant head and neck cancer, especially among men \cite{1,2}. Tobacco and alcohol are two major risk factors for OSCC \cite{3}. Currently, surgery is the primary therapeutic strategy for OSCC, followed by radiotherapy and chemotherapy \cite{4}. Treatments have significantly improved in recent years, but the prognosis of OSCC cases is still unsatisfactory, especially among those in advanced stages, showing a five-year survival rate of less than 50% \cite{5}. Delay in diagnosis, high invasion, and soaring costs for therapy may be partially responsible for dismal clinical outcomes of OSCC \cite{6,7}. And ascertaining molecular etiology of OSCC is of great help for early diagnosis and treatment of this disease. Reportedly, OSCC is a combined result of multiple genetic alterations and environmental factors \cite{8}. So genetic factors play a key role in the pathogenesis of OSCC.

MicroRNAs (miRNAs) are a group of single-strand non-coding RNAs, with a length of about 22 nucleotides \cite{9}. Until now, ~2000 miRNAs have been identified in humans, which could regulate one-third of genes in the genome \cite{10}. Given their regulatory functions on gene expression, miRNAs impose certain influences on a variety of biological pathways \cite{11}. Expression patterns of miRNAs are specific towards different cells, tissues and developmental stages \cite{12}, and their abnormal expressions may cause gene dysregulation, such as translation repression, direct mRNA degradation, and miRNA-mediated mRNA decay, thus resulting in diseases \cite{13}. Dysregulated miRNAs may act as oncogenes or suppressors in tumourigenesis. MiR-638 is a widely studied miRNA, and its down-regulation has been observed in several types of malignancies, such as hepatocellular carcinoma \cite{14}, osteosarcoma \cite{15}, gastric cancer \cite{16}, etc. MiR-638 expression deficiency may contribute to malignant progression. However, the functional roles of miR-638 in OSCC were rarely reported.

In this study, we investigated expression patterns of miR-638 in OSCC tissues and cells. Moreover, cell experiments were carried out to explore the function of miR-638 on OSCC progression, as well as related molecular mechanisms.

Materials and methods

Patients and tissue specimens

A total of 107 OSCC patients who were pathologically confirmed were recruited from the School and Hospital of Stomatolagy, Shandong University. Malignant tissue
specimens and adjacent normal ones were collected from all the cases in surgery, frozen in liquid nitrogen immediately, and then stored at −80 °C. All of the included patients were adults, and none of them had received any pre-operative treatments, such as chemotherapy and radiotherapy.

Clinical characteristics of the included patients were collected and summarized in Table 1. The cases contained 60 males and 47 females, with an average age of 62.08 ± 10.25 years. Of them, 54 had a history of smoking, while 55 had a drinking history. Besides, 45 patients exhibited low differentiation, and lymph node metastasis was observed in 38 ones. According to TNM staging, 65 patients were classified into stages I–II while other 42 into stages III–IV.

The present study was approved by the Ethics Committee of the hospital. All of the patients signed written informed consents.

**Cell line and culture**

OSCC cell lines Tca-8113, SCC-4 and SCC-9 (ATCC® CRL-1629™) and normal human immortalized oral mucosa epithelial cell HOK (human oral keratinocytes, NHOK) (ATCC® PCS-200–014™) were bought from American type culture collection (ATCC). All of the cell lines were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell medium was incubated at 37°C with the presence of 5% CO2.

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from the collected tissue and cell specimens using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc.), and experimental procedures were carried out according to the introduction of the manufacturer. Then cDNA was synthesized using PrimerScript RT reagent kit (Takara, Dalian, China). Quantitative analysis was carried out using the qRT-PCR method adopting SYBR Green PCR master mix (Applied Biosystems, USA) in 7300 Real-Time PCR System (Applied Biosystems, USA). Our used primer sequences were as follows: U6 forward: 5’-CTCGCTTCGGCAGCACA-3’; reverse: 5’-AACGCTTCACGAATTTGCGT-3’, miR-638 forward: 5’-AAGGGATCGCGGGCGGGT-3’; reverse: 5’-CAGTGCAGGGTAGGAGGT-3’, GAPDH forward: 5’-AAGGCTGGGGCTCATTTGCAGG-3’; reverse: 5’-AGTGGTGGTGCAGGAGGCA-3’; phospholipase D1 (PLD1) forward: 5’-AAGGCGGCTCGTGATGTGG-3’; reverse: 5’-ATGGGCTGTTGTTTGAGACTTTGG-3’.

**Western blot analysis**

Western blot analysis was performed to detect expression patterns of related proteins. RIPA Lysis and Extraction Buffer (Thermo Scientific, Waltham, MA, USA) were used for protein isolation. Then, the protein was quantified adopting BCA
Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Same volumes of protein samples were loaded to 10% SDS-PAGE, and then transfected to polyvinylidene fluoride membrane (0.45 μm pore size; EMD Millipore, Billerica, MA, USA) under the condition of 300 mA for 1.5 h. Later, 5% skimmed milk was used to block the membrane at room temperature for 2 h. Subsequently, the membranes were incubated with specific antibodies, respectively, such as anti-β-catenin antibody (1:5000, Abcam), anti-C-myc antibody (1:1000, Abcam), anti-cyclin D1-antibody (1:10,000, Abcam), and anti-GAPDH-antibody (1:10,000, Abcam). GAPDH was employed as a loading control. Then, the membranes were incubated with secondary anti-rabbit IgG antibody (1:2000, Abcam) at room temperature for 2 h. Chemi Genius gel imaging system was used to analyze band gray. Each experiment was repeated three times.

Cell proliferation evaluation

The effects of miR-638 expression levels on cell proliferation were investigated using MTT method. The cells were seeded into 96-well plate with a destiny of 1 × 10^4 cells/mL, and then incubated at 37°C with 5% CO_2_. Later, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added into the cell medium every 24 h (cultured for 0, 24, 48 and 72 h), and incubated for additional 4 h. Afterwards, 150 DMSO was added and incubated at dark for 30 min to stop the reaction. The absorbance at 490 nm of the cell medium was detected using a Microplate Reader (TECAN, Salzburg, Austria) to estimate cell proliferation ability. Each test was performed three times.

Cell motility assay

Transwell analysis (8.0 μm pore size, Costar, Shanghai, China) was performed to estimate cell motility abilities, including migration and invasion. For migration analysis, the upper chamber was filled with 200 μL serum-free DMEM medium, while the lower chamber was coated with 500 μL DMEM medium with 10% FBS. The cells (5 × 10^4 cells/mL) were seeded into the upper chamber, and incubated 37°C with 5% CO_2_. 48h later, the cells in the lower chamber were counted under an inverted microscope (IX31; Olympus Corporation, Tokyo, Japan). Five random files were selected for each sample. In invasion assay, the upper chamber was pre-coated with additional Matrigel (Corning Glass Works, Corning, N.Y., USA).

Luciferase reporter assay

Luciferase reporter assay was performed to explore the targeted relationship between miR-638 and PLD1. In brief, the fragment of PLD1 gene containing the binding site of miR-638 (PLD1-wt), and the fragment with the mutated binding site (PLD1-mt) were amplified through PCR method and cloned to luciferase reporter vector pGL3 (Promega, USA) respectively. Experiment procedures were carried out according to the manufacturer’s instruction. The recombinational luciferase vectors (PLD1-wt and PLD1-mt) combined with miR-638 mimic or mimic were transfected to OSCC cells in vitro using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for 48 h, luciferase activity of the transfected cells was analyzed employing Dual-Luciferase Reporter Assay System (Promega Corporation). Each sample was tested in triplicate.

Statistical analysis

Continuous variables were shown as mean ± standard deviation (SD), and compared between two groups using student's t-test. Categorical data were recorded as case number and percentage, and their comparisons were performed through the chi-square test. All data analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA), and figures were plotted applying GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA). P-values <.05 predicted that the results were statistically significant.

Results

Down-regulation of miR-638 in OSCC tissues and cells

The expression of miR-638 in collected OSCC tissues and cells was detected using qRT-PCR method. As shown in Figure 1, the expression level of miR-638 mRNA in OSCC tissues was significantly decreased compared with corresponding adjacent normal tissues (P < .001, Figure 1(A)). Moreover, miR-638 expression level in OSCC cells Tca-8113, SCC-4 and SCC-9 were all significantly lower than that in HNOK cells (Figure 1(B), P < .05 for all), with lowest figure in SCC-9 (P < .001).

Association of miR-638 expression with clinical characteristics of OSCC patients

The mean values of miR-638 expression in malignant specimens were frequently employed as the cut-off value for grouping in the analysis of the relationship between miR-638 expression and clinical characteristics among cancer patients in previous studies [17,18]. In our study, the patients were divided into high (n = 59) and low (n = 48) expression group based on their mean expression of miR-638 in HCC tissues. Chi-square test was applied to estimate the relationship between miR-638 expression and clinical characteristics of OSCC patients. The results demonstrated that miR-638 expression was negatively correlated with lymph node metastasis (P = .044) and TNM stages (P = .001). Meanwhile, the patterns of miR-638 expression had no close relationship with the patients’ age (P = .131), gender (P = .414), smoking (P = .281), drinking (P = .899), or differentiation (P = .133) (Table 1).

MiR-638 inhibited OSCC cell proliferation, migration and invasion

In order to investigate functional roles of miR-638 in OSCC progression, miR-638 mimic, miR-638 inhibitor, as well as
corresponding negative controls were designed and transfected to OSCC cells in vitro. QRT-PCR method was performed to estimate transfection efficacy. Analysis results demonstrated that compared to the controls, the transfection of miR-638 mimic could significantly enhance the expression of miR-638 (P<.01), while miR-638 inhibitor could suppress miR-638 expression in OSCC cells (P<.05) (Figure 2(A)).

Biological behaviors of the transfected cells were detected. MTT assay suggested that enforced expression of miR-638 using mimic transfection could significantly suppress cell proliferation (P<.05); moreover, the knockdown of miR-638 enhanced proliferation ability of OSCC cells (P<.05) (Figure 2(B)). Furthermore, restored expression of miR-638 imposed inhibiting effects on cell migration and invasion, while the transfection of miR-638 inhibitor could promote cell migration and invasion, according to transwell analysis (P<.05 for all) (Figure 2(C,D)).

**PLD1 was a potential target of miR-638 in OSCC**

Bioinformatics analysis implemented through TargetScan Human prediction software demonstrated that the 3’UTR of PLD1 gene had complementary sequences of miR-638 (Figure 3(A)). Moreover, the study carried out in gastric cancer also revealed a targeted relationship between PLD1 and miR-638 [16]. Thus, we speculated that PLD1 was a potential target of miR-638 in OSCC. Luciferase reporter assay demonstrated that in cells transfected by PLD1-wt, the presence of miR-638 mimic could significantly reduce luciferase activity of the cells (P<.01), while the co-transfection with miR-638 and PLD1-mt had no obvious influences on luciferase activity of the cells (P>.05) (Figure 3(B)). MiR-638 could bind to the 3’UTR of PLD1 gene in OSCC.

QRT-PCR method was used to investigate the relative expression of PLD1 mRNA in OSCC. The results suggested that the levels of PLD1 mRNA were significantly increased in OSCC cells, compared to non-cancerous cells (P<.001) (Figure 4(A)). Furthermore, enforced expression of miR-638 could obviously suppress PLD1 expression (P<.01), while miR-638 inhibition up-regulated PLD1 in OSCC (P<.01) (Figure 4(B)). MiR-638 could negatively regulate the expression of PLD1 in OSCC. PLD1 was a target of miR-638 in OSCC.

**MiR-638 could regulate the activity of wnt/β-catenin signalling pathway**

Western blot analysis was performed to investigate the expression of the proteins in wnt/β-catenin signalling pathway for the transfected cells. As displayed in Figure 5, compared to the controls, the levels of c-Myc, β-catenin and Cycline D1 were significantly decreased after the transfection of miR-638 mimic, while the transfection of miR-638 inhibitor could obviously constrain the expression of the proteins (P<.05 for all).

**Molecular mechanisms of miR-638 in the progression of OSCC**

In order to investigate potential mechanisms underlying functional roles of miR-638 in OSCC progression, we designed si-PLD1 vector to knockdown PLD1 in cells transfected by miR-638 inhibitor, and cells transfected by miR-638 inhibitor served as controls. Western blot analysis suggested that compared to the controls, the co-transfection with miR-638 inhibitor and si-PLD1 could down-regulate c-Myc, β-catenin and Cycline D1, revealing the inactivity of wnt/β-catenin signalling pathway (P<.05 for all) (Figure 6).

Biological behaviors of OSCC cells transfected by miR-638 inhibitor and si-PLD1 were also investigated in the current study. MTT assay suggested that the co-transfection with miR-638 inhibitor and si-PLD1 decreased cell proliferation ability (P<.01) (Figure 7(A)). Transwell analyses demonstrated that the presence of si-PLD1 could obviously suppress the migration (Figure 7(B)) and invasion (Figure 7(C)) of OSCC cells transfected by miR-638 inhibitor (P<.05 for both).

**Discussion**

miRNAs play important roles in tumourigenesis through their target genes. Exploring their function and related molecular
mechanisms may provide new insight into the etiology of cancers. In the current study, we analyzed the functional roles of miR-638 in OSCC progression, as well as, potentially related molecular mechanisms. We found that the expression of miR-638 was significantly decreased in OSCC tissues and cells; moreover, its down-regulation predicted positive lymph node metastasis and advanced TNM stages. The expression of miR-638 was negatively correlated with malignant behaviors of OSCC cells in vitro. MiR-638 could suppress the wnt/β-catenin signalling pathway through PLD1, thus inhibiting OSCC progression.

OSCC is a frequently diagnosed malignancy around the world, with poor five-year survival [19]. And exploring molecular mechanisms underlying OSCC progression is crucial to improve its treatments and prognosis. MicroRNAs, gene expression regulators, are involved in various types of cancers, including OSCC [20]. Dysregulated miRNAs are reportedly to act as tumour suppressors or oncogenes in OSCC origination and progression. In the current study, we found that the expression of miR-638 was significantly down-regulated in OSCC tissues and cells; moreover, its decreased expression predicted positive lymph node metastasis and advanced TNM stages. MiR-638 expression showed a negative correlation with OSCC aggressive progression. Furthermore, cell experiments demonstrated that enforced expression of miR-638 could inhibit malignant proliferation, migration and

Figure 2. The transfection with miR-638 mimic could enhance the expression of miR-638, while transfection with miR-638 inhibitor down-regulated miR-638 (A). Enforced miR-638 could significantly suppress OSCC cells’ proliferation (B), migration (C) and invasion (D), and the knockdown of miR-638 promoted malignant behaviors of OSCC cells. **P < .01; *P < .05.
invasion of OSCC cells, while its knockdown might enhance aggressive biological behaviors of OSCC cells. All of the data revealed that miR-638 acted as a tumour suppressor in OSCC. The present study might be the first one investigating the functional roles of miR-368 in OSCC. Our findings were in consistent with those from studies on other types of cancers. Shen et al. reported that miR-638 as a tumour suppressor played pivotal roles in gastric cancer [21]. Shi et al. suggested that hepatocellular carcinoma patients with low expression of serum exosomal miR-638 were more likely to undergo poor prognosis. Moreover, the proliferation ability of hepatocellular carcinoma cells was inhibited after the transfection with miR-638 mimic [22]. Taken together, miR-638 played an inhibitory role in tumourigenesis and thus might be employed as a therapeutic target.

MiRNAs possess no capability to code protein, and they take part in biological processes through their target genes [23]. In the current study, PLD1 was confirmed as a target gene of miR-638, according to bioinformatics analysis and luciferase reporter assay. PLD1 expression was negatively regulated by miR-638. Such conclusion was in line with that from the study of Zhang et al [16]. In their study, PLD1 was confirmed as a target of miR-638 as well, and miR-638

![Figure 3](image1.png)

Figure 3. Bioinformatics analysis demonstrated that the 3’UTR of PLD1 gene had complementary sequences of miR-638 (A). In cells transfected by PLD1-wt, the presence of miR-638 mimic could significantly reduce luciferase activity of the cells, while the co-transfection with miR-638 and PLD1-mt had no obvious influences on luciferase activity of the cells (B). **P < .01.

![Figure 4](image2.png)

Figure 4. The expression of PLD1 was significantly up-regulated in SCC-9 cells, compared to non-cancerous HPK cell line (A). Moreover, enforced expression of miR-638 could obviously suppress the expression of PLD1, while miR-638 inhibition up-regulated PLD1 in OSCC (B). ***P < .001; **P < .01.

![Figure 5](image3.png)

Figure 5. Western blot images for the expressions of wnt/β-catenin pathway-related proteins in transfected cells (A). Quantitative analysis for western blot results (B). **P < .01; *P < .05.
inhibited the progression of gastric cancer through regulating PLD1 expression. PLD1 belongs to PLD lipid-signalling enzyme super-family, which represents a major source of signal-activated phosphatidic acid (PtdOH) generation for numerous cell-surface receptors [24]. PLD1 is generally accepted as an oncogene, and its up-regulation promotes angiogenesis and metastasis in tumourigenesis [25]. Oncogenic function of PLD1 has been reported in multiple malignancies, including glioma [26], hepatocellular carcinoma [27], breast cancer [28], etc. In the current study, we confirmed that miR-638 played an anti-tumour role in OSCC through its negative regulation on PLD1.
Several published articles have demonstrated that PLD1 could activate wnt/β-catenin in cancer progression [29–31]. Based on these articles, we speculated that miR-638 might regulate the wnt/β-catenin pathway through PLD1. In our study, we found that enforced expression of miR-638 could inactivate wnt/β-catenin, while miR-638 knockdown resulted in excessive activation of the pathway. PLD1 can reverse the function of miR-638 on wnt/β-catenin pathway, and inhibitory function of miR-638 on wnt/β-catenin pathway is dependent on PLD1. The present study might be the first one discussing the functional roles of miR-638 in OSCC progression and related molecular mechanisms. Despite those encouraging results, several limitations in this work should be stated. Firstly, the sample size was relatively small, which might influence the statistical power of our results. Secondly, the function of miR-638 was proved only in one OSCC cell line due to the limited study period. Expression profiles and function of miR-638 in other OSCC cell lines remained unclear. SCC-9 is a tongue squamous cell carcinoma cell line which was derived from a 25-year-old male, and shows high colony formation ability in vitro and oncogenicity in vivo [32]. This cell line is frequently used for OSCC experiments in vitro. Thus, the results obtained in our study had certain representativeness. Thirdly, animal models are required to verify our results. Besides, apart from wnt/β-catenin pathway, miR-638 might take part in OSCC progression through multiple signalling pathways. Therefore, further investigations should be designed to explore regulation through netweb for miR-638 in OSCC.

In conclusion, the expression of miR-638 is down-regulated in OSCC, and shows a negative correlation with cancer progression. MiR-638 can inhibit the proliferation, migration, and invasion of OSCC cells. The anti-tumour action of miR-638 in OSCC may be dependent on miR-638/wnt/β-catenin axis.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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