MicroRNA-655 suppresses cell proliferation and invasion in oral squamous cell carcinoma by directly targeting metadherin and regulating the PTEN/AKT pathway

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Abstract. MicroRNAs (miRNAs) are important regulators of a variety of biological processes and their dysregulation is closely related to cancer formation and progression. Therefore, examination of aberrantly expressed miRNAs in oral squamous cell carcinoma (OSCC) may provide important clues for the diagnosis and treatment of patients with OSCC. The aim of the present study was to determine miRNA (miR)-655-3p expression in OSCC tissues and cell lines, and to investigate the biological roles and mechanisms of miR-655-3p associated with OSCC. Data from the present study indicated that miR-655 expression was significantly downregulated in human OSCC tissues and cell lines. Overexpression of miR-655 attenuated cell proliferation and invasion in OSCC in vitro. Metadherin (MTDH) mRNA was predicted as a potential target of miR-655 by bioinformatics analysis, and this was confirmed by luciferase reporter assay, reverse transcription-quantitative polymerase chain reaction and western blot analysis. In OSCC tissues, MTDH was highly expressed and inversely correlated with miR-655 expression levels. MTDH overexpression reversed the inhibitory effects of miR-655 mimics in OSCC cells. Notably, the upregulation of miR-655 expression inhibited the activation of the phosphatase and tensin homolog (PTEN)/RAC-α serine/threonine-protein kinase (AKT) pathway in OSCC cells. Therefore, these results may provide the first evidence that miR-655 targets MTDH to inhibit proliferation and invasion of OSCC by inhibiting PTEN/AKT signaling. Thus, the restoration of miR-655 expression may be a novel therapeutic strategy for patients with OSCC.

Introduction

Oral cancer is the sixth most common malignant neoplasm worldwide (1); oral squamous cell carcinoma (OSCC) is the most common type of oral cancer and accounts for ~90% of all oral cancer cases, with an estimated 300,000 new cases diagnosed per year (2). Human papillomavirus infection, alcohol consumption and smoking were identified as major risk factors for OSCC (3). Currently, the main effective treatments for patients are radical operation in combination with radiotherapy, neoadjuvant chemotherapy and targeted therapy (4). Despite remarkable improvements in diagnostic strategies and surgery, the clinical outcome of patients diagnosed with OSCC remains unsatisfactory, with a 5-year survival rate of <50% (5). High local recurrence rates and metastasis are largely responsible for the poor prognosis of patients with OSCC (6). Therefore, further elucidation of the mechanisms underlying the pathogenesis of OSCC may aid the development of tumor-specific biomarkers and novel therapeutic methods for early diagnosis and therapy of patients with this malignancy.

miRNAs (miR) are a class of endogenous, highly conserved, noncoding, short RNAs that serve a role in gene regulation (7). miRNAs interact directly with the 3′-untranslated region (UTR) of their target mRNAs in a base-pairing manner and induce mRNA degradation and/or inhibit transcription (8). A miRNA may regulate the expression of various mRNAs simultaneously; it has been estimated that the expression of ~67% of all human protein coding genes are modulated by miRNAs (9). Previous studies have reported that abnormally expressed miRNAs may serve a role in a number of human disorders, particularly cancers (10). For example, abnormal miRNA expression was reported in OSCC (11), bladder cancer (12), gastric cancer (13), thyroid cancer (14) and lung cancer (15). Dysregulated miRNAs may serve as oncogenes or tumor suppressors, and are involved in the regulation of numerous cellular biological processes, including cell proliferation, apoptosis, invasion, metastasis, angiogenesis and epithelial-mesenchymal transition (16-18). Therefore, miRNAs may serve as novel, effective biomarkers for cancer diagnosis, therapy and prognosis.

miRNA-655-3p (miR-655), which is located on chromosome 14q32, was previously reported to be aberrantly expressed in multiple human cancers, including hepatocellular...
carcinoma (19,20), triple-negative breast cancer (21) and esophageal squamous cell carcinoma (22). However, the expression, role and molecular mechanisms of miR-655 in OSCC have not yet been elucidated. Therefore, the aim of the present study was to detect miR-655 expression in OSCC tissues and cell lines, and to investigate its biological roles in OSCC. The mechanisms underlying the involvement of miR-655 in OSCC were also investigated. Bioinformatics analyses were performed to determine potential targets of miR-655, and the results revealed that metadherin (MTDH) was a candidate target of miR-655. Subsequent experiments were performed to determine whether MTDH was a direct target gene of miR-655 in OSCC cells. MTDH has previously been reported to contribute to the regulation of the phosphatase and tensin homolog (PTEN)/protein kinase (AKT) signaling pathway (23,24); therefore, the present study also investigated whether miR-655 participated in the regulation of the PTEN/AKT signaling pathway in OSCC cells.

Materials and methods

Collection of OSCC tumoral tissues. A total of 26 pairs of OSCC tissues and adjacent non-tumoral oral tissues were collected from patients diagnosed with OSCC and treated with radical surgery at Yidu Central Hospital of Weifang (Weifang, China) between November 2014 and February 2017. All patients (15 males and 11 females; aged 49-72 years old) enrolled in the study received no radiotherapy, chemotherapy, targeted therapy or other treatments prior to surgery. Patients treated with radiotherapy, chemotherapy, targeted therapy or other treatments prior to surgical resection was excluded from the present study. All tissues were quickly frozen in liquid nitrogen following surgery and stored in liquid nitrogen until used in subsequent experiments. The present study was approved by the Ethics Committee of Yidu Central Hospital of Weifang, and written informed consent was provided by all patients prior to sample collection.

Cell culture and transfection assay. The OSCC cell lines Tca8113, CAL-27 and SCC-9 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Primary normal human oral keratinocytes (NHOK) were acquired from ScienCell Research Laboratories, Inc. (San Diego, CA, USA) and grown in the oral keratinocyte medium (ScienCell Research Laboratories, Inc.). All cell lines were maintained at 37˚C in a humidified chamber with 5% CO₂.

miR-655 mimics and mimic negative-controls (miR-NC) were synthesized by Guangzhou Ruibo Biological Technology, Co., Ltd. (Guangzhou, China). The miR-655 mimic sequence was 5'-UUC UCC GAA CGU GUC ACG UTT-3'. MTDH overexpression vector (pCMV-MTDH) and empty pCMV vector were produced by Shanghai GenePharma Co., Ltd. (Shanghai, China). For transfection assays, cells (5x10⁵ cells/well) were plated onto 6-well plates and transfected with miR-655 mimics (100 pmol), miR-NC (100 pmol), pCMV-MTDH (4 µg) or empty pCMV vectors (4 µg) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature, according to the manufacturer's protocol. Following transfection for 24 h, CCK-8 assay was performed. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and cell invasion assays were performed at a total of 48 h post-transfection. Western blot analysis was used to detect protein expression in transfected cells a total of 72 h post-incubation.

RNA isolation and RT-qPCR. RT-qPCR was performed to determine the expression levels of miR-655 and MTDH mRNA. Total RNA was extracted from tissue samples (100 mg) or cells (1x10⁶) using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To detect miR-655 expression, reverse transcription was carried out using a TaqMan miRNA Reverse Transcription kit followed by qPCR with a TaqMan miRNA assay kit (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). The temperature protocol for RT was as follows: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. The thermocycling conditions used for qPCR were as follows: 50˚C for 2 min and 95˚C for 10 min; followed by 40 cycles of denaturation at 95˚C for 15 sec; and subsequently annealing/extension at 60˚C for 60 sec. For the quantification of MTDH mRNA levels, a PrimeScript RT Reagent kit was used to synthesize cDNA, and qPCR was performed using SYBR Premix Ex Taq II (both from Takara Biotechnology Co., Ltd., Dalian, China). The temperature protocol for RT was as follows: 37˚C for 15 min and 85˚C for 5 sec. The thermocycling conditions used for qPCR were as follows: 5 min at 95˚C; followed by 40 cycles of 95˚C for 30 sec and 65˚C for 45 sec. RT-qPCR was performed in an ABI PRISM 7000 Fluorescent Quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 small nuclear RNA and GAPDH were used as the endogenous controls to normalize the levels of miR-655 and MTDH mRNA, respectively. The primers used were as follows: miR-655 forward, 5'-TCCGAAACATGGTTAA-3' and reverse, 5'-GTGCGAGGTCGGAGGT-3'; U6 forward, 5'-CTC GCTTCGCGACGACA-3' and reverse, 5'-AAGGTTCCTACGAGTTTGCG-3'; MTDH forward, 5'-GGG-3' and reverse, 5'-CAGGAAATGATGCGGTG-3'; and GAPDH forward, 5'-CGGAGTCAAGGTTGTGTCG TC-3' and reverse, 5'-AGCCTTCTCCTCATGTTGAA GAC-3'. Relative gene expression was calculated using the 2⁻ΔΔCq method (25).

MTT cell proliferation assay. Following transfection for 24 h, cells (2,000 cells/well) were seeded into 96-well plates, and the extent of proliferation was detected at room temperature by performing an MTT assay at 0, 24, 48 and 72 h. Briefly, a total of 20 µl MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well. The culture plates were incubated at 37˚C for 4 h. Subsequently, the supernatant containing the MTT solution was discarded and 150 µl dimethyl-sulfoxide was added into each well to dissolve the formazan precipitate. The absorbance was read at a wavelength of 490 nm using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell invasion assay. Transfected cells were trypsinized, collected and washed with FBS-free DMEM/F-12 medium at 48 h post-transfection. A total of 1x10⁵ cells were suspended in 200 µl FBS-free DMEM/F-12 medium and seeded in the
upper Transwell chambers (8 µm; Corning Inc., Corning, NY, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). DMEM/F-12 medium (500 µl) containing 10% FBS was added into the lower chamber. Following 24 h incubation at 37°C with 5% CO₂, the non-invading cells on the upper membrane were scraped off with cotton swabs. The invasive cells were fixed with 95% ethanol for 15 min and stained with 0.5% crystal violet for 15 min, both at room temperature. The number of invasive cells was counted in at least five randomly selected fields using an IX51 inverted microscope (magnification, x200; Olympus Corporation, Tokyo, Japan).

Bioinformatics analysis. TargetScan (version 7.2; http://www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de) were used to predict the potential targets of miR-655.

Luciferase report assay. Human MTDH 3’-UTRs containing putative wild-type (WT) or mutated (Mut) binding sites for miR-655 were amplified by Shanghai GenePharma Co., Ltd., cloned into the psiCHECK-2 reporter vector (Promega Corporation, Madison, WI, USA), and designated MTDH-3’-UTR-WT or MTDH-3’-UTR-Mut. Cells were seeded into 24-well plates (1.5x10⁴ cells/well) 1 day prior to transfection and incubated at 37°C with 5% CO₂, miR-655 mimics (50 pmol) or miR-NC (50 pmol) were co-transfected into cells with either MTDH-3’-UTR-WT (100 ng) or MTDH-3’-UTR-Mut (100 ng) using Lipofectamine 2000, and incubated at 37°C with 5% CO₂ for 48 h, according to the manufacturer's protocol. Luciferase activities were evaluated using the Dual Luciferase Assay kit (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activities were normalized to Renilla luciferase activities.

Western blot analysis. The Total Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was used to extract total protein from tissue samples (200 mg) or cells (1x10⁶). Subsequently, the concentration of total protein was quantified using a Bicinchoninic Acid Assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Equivalent amounts of protein (30 µg) were separated by 10% SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 1 h and incubated with the following primary antibodies overnight at 4°C: Mouse anti-human MTDH antibody (1:1,000; cat. no. sc-517220; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-human PTEN antibody (1:1,000; cat. no. ab77161; Abcam, Cambridge, UK), mouse anti-human RAC-α serine/threonine-AKT antibody (1:1,000; cat. no. sc-56878), mouse anti-human phosphorylated (p)-AKT antibody (1:1,000; cat. no. sc-271966; both Santa Cruz Biotechnology, Inc.) and mouse anti-human GAPDH antibody (1:1,000; cat. no. ab125247; Abcam). Membranes were washed three times with TBST, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000; cat. no. ab205719; Abcam). Following extensive washes with TBST, protein signals were visualized with an Enhanced Chemiluminescence Detection System (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as a loading control for normalization of protein expression levels. Densitometric analysis was performed using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation, based on the results of at least three repeated experiments. Analyses were performed with SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). Differences between two groups were analyzed with a two-tailed Student’s t-test. One-way analysis of variance followed by Student-Newman-Keuls post hoc test was performed to investigate the differences between more than two groups. Spearman’s correlation analysis was used to examine the correlation between miR-655 and MTDH mRNA expression levels in OSCC tissues. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-655 expression is downregulated in OSCC tissues and cell lines. To examine the role of miR-655 in OSCC, miR-655 expression levels were determined in a total of 26 pairs of OSCC tissues and adjacent non-tumoral tissues using RT-qPCR. Compared with expression in the adjacent non-tumoral tissues, miR-655 expression was significantly lower in OSCC tissues (Fig. 1A; P<0.05). In addition, miR-655
expression levels were examined in three OSCC cell lines (Tca8113, CAL-27 and SCC-9) and NHOK cells. The data indicated that miR-665 expression was significantly lower in OSCC cell lines compared with expression in NHOK cells (Fig. 1B; P<0.05). These results suggested that the expression of miR-665 is significantly reduced in OSCC; this reduced expression may be related to OSCC progression.

**miR-665 inhibits Tca8113 and CAL-27 cell proliferation and invasion.** Tca8113 and CAL-27 cells exhibited relatively low miR-665 expression among the three OSCC cell lines; therefore, these cells were selected for subsequent experiments. To explore the effects of miR-665 in OSCC, miR-665 mimics were transfected into Tca8113 and CAL-27 cells to increase miR-665 expression levels. RT-qPCR analysis confirmed that miR-665 was significantly increased in Tca8113 and CAL-27 cells transfected with miR-665 mimics compared expression levels in cells transfected with miR-NC (Fig. 2A; P<0.05).

MTT and cell invasion assays were performed to examine the effects of miR-665 overexpression on proliferation and invasion, respectively, of Tca8113 and CAL-27 cells. The results indicated that transfection with miR-665 mimics led to a significant reduction in proliferation and invasion compared with the respective miR-NC groups (Fig. 2B and C, respectively; P<0.05). These results suggested that miR-665 may have tumor suppressive roles in OSCC growth and metastasis.

**miR-665 directly targets and downregulates MTDH mRNA expression in OSCC cells.** The biological roles of miRNAs in human malignancies depend on their specific targets; therefore, bioinformatics analysis was performed to predict the potential targets of miR-665. A total of 780 conserved sites were revealed, including A disintegrin metallopeptidase domain-containing protein 10; pituitary tumor-transforming 1 interacting protein; and membrane associated guanylate kinase, WW and PDZ domain containing 2. Among these candidates, MTDH was predicted as a major target of miR-665 (Fig. 3A), which has been previously reported to be involved in OSCC occurrence and development [26-29]. To confirm this hypothesis, luciferase reporter assays were performed to determine whether the 3'-UTR of MTDH was directly targeted by miR-665 in OSCC cells. Luciferase activity was significantly reduced in MTDH-3'-UTR-WT + miR655 mimics transfected Tca8113 and CAL-27 cells compared with cells co-transfected with MTDH-3'-UTR-WT + miR-NC (Fig. 3B; P<0.05), whereas no significant differences in luciferase activities were identified in cells co-transfected with miR-665 mimics or miR-NC and MTDH-3'-UTR-Mut. Furthermore, RT-qPCR and western blot assays demonstrated that overexpression of miR-665 significantly reduced the expression levels of MTDH mRNA and protein (Fig. 3C and D, respectively; P<0.05) in Tca8113 and CAL-27 cells compared with miR-NC transfected cells. These data indicated that MTDH is a direct target of miR-665 in OSCC cells.
miR-655 expression is inversely correlated with MTDH mRNA expression levels in OSCC tissues. To further explore the association between miR-655 and MTDH in OSCC, MTDH mRNA expression levels were determined in the 26 pairs of OSCC tissues and adjacent non-tumoral tissues. RT-qPCR analysis revealed that MTDH mRNA expression was significantly higher in OSCC tissues compared with the expression levels in the adjacent non-tumoral tissues (Fig. 4A; P<0.05). In addition, western blot analysis was performed to detect MTDH protein levels in OSCC tissues and adjacent non-tumoral tissues, which demonstrated that the protein expression level of MTDH was notably higher in OSCC tissues compared to that in adjacent non-tumoral tissues (Fig. 4B). Furthermore, the expression of miR-655 exhibited an inverse correlation with MTDH mRNA expression in OSCC tissues (Fig. 4C; r=-0.5949; P<0.05). These results further indicated that MTDH may be a novel target of miR-655 in OSCC.
**Inhibiting MTDH is crucial for the inhibitory effects of miR-655 expression in OSCC cells.** Rescue experiments were performed to further determine whether the suppressive roles of miR-655 were mediated through MTDH in OSCC cells. The MTDH overexpression vector pCMV-MTDH or the pCMV empty vector was transfected into Tca8113 and CAL-27 cells. RT-qPCR analysis was performed to confirm that MTDH mRNA expression was significantly increased in Tca8113 and CAL-27 cells that were transfected with pCMV-MTDH compared with pCMV empty vector-transfected cells (Fig. 5A; P<0.05). To perform rescue experiments, Tca8113 and CAL-27 cells were co-transfected with pCMV-MTDH or pCMV empty vector along with miR-655 mimics. Western blot analysis revealed that the MTDH protein expression that was decreased by miR-655 mimics was restored in cells co-transfected with pCMV-MTDH (Fig. 5B; P<0.05). Subsequent MTT and cell invasion assays were conducted to evaluate the effects on cell proliferation and invasion in Tca8113 and CAL-27 cells co-transfected with miR-655 mimics and either pCMV or pCMV-MTDH. These results suggested that miR-655 may inhibit the activation of the PTEN/AKT signaling pathway in OSCC through the negative regulation of MTDH expression.

**Discussion**

Numerous studies have indicated that miRNAs are important regulators for a variety of biological processes, and their dysregulation is closely related to cancer formation and progression (30-32). Therefore, the identification of aberrantly expressed miRNAs may provide important insight into the diagnosis and therapy of patients with OSCC. In the present study, miR-655 expression in OSCC was investigated as well as the roles and underlying mechanisms associated with miR-655 in the progression of OSCC. To the best of our knowledge, the present study is the first to demonstrate that miR-655 may have exhibited tumor suppressive roles in OSCC by directly targeting MTDH and regulating the PTEN/AKT signaling pathway, which suggested that miR-655 may represent an effective therapeutic agent in the treatment of patients with OSCC.

In the present study, miR-655 expression was demonstrated to be significantly lower in OSCC tissues and cell lines compared with expression levels in adjacent non-tumor tissues and NHOK cells. miR-655 has been previously reported to be
Many direct targets of miR-655 have been validated, including ADAM metallopeptidase domain 10 in hepatocellular carcinoma (20), paired-related homeobox 1 in triple-negative breast cancer (21), pituitary tumor-transforming 1 in esophageal squamous cell carcinoma (22) and membrane-associated guanylate kinase, WW and PDZ domain-containing 2 in lung adenocarcinoma (34). In the present study, MTDH was identified as a direct target gene of miR-655 in OSCC. MTDH is located on chromosome 8q22 (35); it has been reported to be highly expressed in various types of human cancer, including glioma (36), breast cancer (37), gastric cancer (38), bladder cancer (39) and cervical cancer (40). In OSCC, both mRNA and protein levels of MTDH were overexpressed in tumor tissues and their overexpression was positively correlated with differentiation, clinical stage, T classification and lymph node metastasis. OSCC patients with high MTDH levels exhibited shorter overall survival rates relative to those patients with low MTDH expression (26,27). In addition, MTDH was previously confirmed as an independent prognostic factor for overall survival rates in OSCC patients (26,27). Highly expressed MTDH was reported to serve important roles in the onset and the progression of OSCC by affecting tumor cell growth, colony formation, migration and invasion (26,28,29). MTDH has previously been reported to be involved in the regulation of the PTEN/AKT signaling pathway (23,24). It has been well established that activation of the PTEN/AKT pathway contributes to OSCC development (41,42). In the present study, it was demonstrated that restoration of MTDH expression inactivated the PTEN/AKT pathway in OSCC cells via regulation of MTDH. These findings suggested that MTDH is an effective candidate for molecular targeted therapy for OSCC.

To the best of our knowledge, the present study is the first to demonstrate that miR-655 expression was reduced in OSCC tissues and cell lines. In addition, miR-655 overexpression suppressed cell proliferation and invasion in OSCC by directly targeting MTDH and regulating the PTEN/AKT pathway. These results may improve our understanding of OSCC pathogenesis and may also provide a theoretical basis for the identification of miR-655 as a potential tumor suppressor in OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HJ and QW designed the research; QW, LL and YL performed functional experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Yidu Central Hospital of Weifang (Weifang, China) and was performed in accordance with The Declaration of Helsinki. Ethics approval and consent to participate in the present study. Written informed consent was obtained from all patients for the use of their clinical tissues, prior to enrolment in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


