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Original Article

MicroRNA-506 suppresses growth and metastasis of oral squamous cell carcinoma via targeting GATA6

Li Deng, Hui Liu

Department of Stomatology, Jining NO. 1 People's Hospital, Jining 272011, Shandong, China

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Abstract: Oral squamous cell carcinoma (OSCC) remains the main cause of cancer mortality, accounts for 90% of oral cancers. Increasing evidence reveals that aberrant expression of microRNA contributes to the development and progression of OSCC, but the roles of microRNA-506 (miR-506) in OSCC remain elusive. Here, we found that the expression of miR-506 was decreased in 21 OSCC tissues and SCC-4 and SCC-9 cells. miR-506 overexpression suppressed proliferation, migration and invasion capabilities of OSCC cells. Moreover, miR-506 overexpression suppressed the luciferase activity of the GATA6-Wt 3'-UTR and decreased the mRNA and protein levels of GATA6. GATA6 overexpression attenuated the suppressive effects of miR-506. Furthermore, GATA6 was increased in OSCC tissues compared with the adjacent normal tissues and inversely associated with miR-506. Taken together, these results suggest that miR-506 is an important suppressor and could be a novel prognostic factor and therapeutic target for OSCC patients.

Keywords: GATA6, growth, metastasis, miR-506, oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) accounts for about 90% of oral cancers, and is widely recognized as the most prevalent cancer worldwide [1, 2]. Despite significant improvements in therapeutic modalities, 5-year overall survival rate of OSCC patients is approximately 50% [3]. Recent studies reveal that early detection is paramount for improving survival rate and prognosis for OSCC patients [4]. Therefore, it is urgent to understand the basic molecular mechanisms driving oral tumorigenesis and screen potential biomarkers for the early detection of OSCC.

Accumulating evidence highlights that microRNAs (miRNAs) are involved in the initiation and progression of many types of cancers, including OSCC [5, 6]. miRNAs are a group of endogenous and short noncoding RNAs of approximately 22 nucleotides in length, which transcriptionally or post-transcriptionally suppress their expression at the mRNA or protein levels through base pairing with the 3'-untranslated region (UTR) of their target mRNAs [7]. Up to now, 2603 different human mature miRNAs have been identified and released in the miRBase database (http://www.mirbase.org/). A variety of miRNAs have been shown to be dysregulated in OSCC, such as miR-126, miR-21 and miR-222, each of which may contribute to the development and progression of OSCC [8-10]. miR-506 functions as a tumor suppressor in several cancers [11-13], however, the detailed roles of miR-506 in OSCC remain largely unknown.

In this study, we found that miR-506 was significantly decreased in OSCC tissues and SCC-4 and SCC-9 cells compared with normal tissues and cells, and inhibitory effects of miR-506 on proliferation, migration, and invasion were investigated. Our results suggest that miR-506 might suppress growth and metastasis of OSCC cells partially by targeting GATA binding protein 6 (GATA6), which is a member of the GATA family of zinc-finger transcription factors.

Materials and methods

Tissue samples and cell lines

A total of 21 pairs of matched OSCC and adjacent non-cancerous tissues were surgically ob-

Table 1. Patient and Tumor Characteristics

Patient and Tumor Characteristics	Cases, n (%)	miR-506 (ΔCt)	GATA6 (ΔCt)
Age (years)			
Mean ± SD	52.17±14.37	1.81 ± 0.50	10.69 ± 3.37
Gender			
Male	14 (66.7)	1.85 ± 0.53	10.60 ± 2.28
Female	7 (33.3)	1.73 ± 0.48	10.90 ± 5.12
Histology/differentiation			
Well	11 (52.4)	2.05 ± 0.52	8.09 ± 2.16
Moderate	7 (33.3)	1.67 ± 0.26	12.87 ± 1.40
Poor	3 (14.3)	1.26 ± 0.35	15.10 ± 0.61
TNM stage			
I	4 (19.1)	2.44 ± 0.46	6.15 ± 1.47
II	5 (23.8)	2.08 ± 0.28	8.74 ± 1.67
III	7 (33.3)	1.64 ± 0.21	11.78 ± 1.53
IV	5 (23.8)	1.28 ± 0.27	14.73 ± 0.73

tained from our department between January 2011 and December 2013. Surgically resected specimens were immediately frozen in liquid nitrogen and stored at -80°C. Both tumor and normal tissues were histologically confirmed by two independent pathologists. TNM classification was defined according to the American Joint Committee on Cancer. The clinical characteristics of the patients were summarized in **Table 1**. Normal oral mucosa cells were scraped using a toothpick from 11 noncancerous volunteers' mouth. Informed consent was obtained from each patient and the research protocol was approved by the Hospital Ethical Committee.

SCC-4 and SCC-9 human OSCC cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human Embryonic Kidney (HEK)-293 cells, as model cells, were obtained from the Chinese Science Institute and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. All cells were incubated at 37°C with 5% CO_2.

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

Total RNA was isolated from the cells and frozen tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. All reagents for qRT-PCR were ordered from Takara (Dalian, China). For GATA6 mRNA expression, the first-strand cDNA was generated using the PrimeScript RT reagent Kit according to the manufacturer's protocol. For miR-506 expression, total RNA was reversely transcribed using miRNA-specific primers for quantification of miR-506. qRT-PCR was performed using the Applied Biosystems 7500 thermocycler (Applied Biosystems) with SYBR Premix Ex Taq II. U6 and β-actin were used as controls for miRNA and mRNA levels, respectively. Relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method, in which $\Delta\Delta Ct = (Ct_{miR\text{-}506} - Ct_{U6})_{oscc} - (Ct_{miR\text{-}506} - Ct_{U6})_{control}$ for miR-506; $(Ct_{GATA6} - Ct_{\beta\text{-}actin})_{oscc} - (Ct_{GATA6} - Ct_{\beta\text{-}actin})_{control}$ for GATA6. The following primers were used in the qRT-PCR analysis: RT: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCTACTC-3', forward: 5'-ATCCAGTGCGTGTCGTG-3' and reverse: 5'-TGCTTAAGGCACCCTTCT-3' for miR-506. Forward: 5'-CCACAGGCAGGTTGGTTTAC-3' and reverse: 5'-CGGCTCCAAAAGGACTATGC-3' for GATA6. U6 and β-actin were used to normalize miRNA and mRNA, respectively. All reactions were carried out in triplicate.

Plasmids and transfection

Pre-miR-506 was synthesized (SangonBiotech, Shanghai, China) and inserted into pcDNA™ 6.2-GW vector at EcoRI and HindIII sites (Promega) with the sense strand 5'-AATTCGCCAC CACCATCAGCCATACTATGTGTAGTGCCTTATTCAGGAAGGTGTTACTTAATAGATTAATATTTGTAAGGCACCCTTCTGAGTAGAGTAATGTGCAACATGGACAACATTTGTGGTGGCA-3' and antisense strand 5'-AGCTTGCCACCACAAATGTTGTCCATGTTGCACATTACTCTACTCAGAAGG TGCCTTACAAATATTAATCTATTAAGTAACACCTTC CTGAATAAGGCACTACACATAGTATGGCTGATG GTGGTGGCG-3'. The complimentary sequence of GATA6 3'-UTR for miR-506 (GATA6-Wt) and mutated 3'-UTR sequence (GATA6-Mut) were synthesized and cloned into pmirGLO vector at Sac I and Xho I sites (Promega). The GATA6

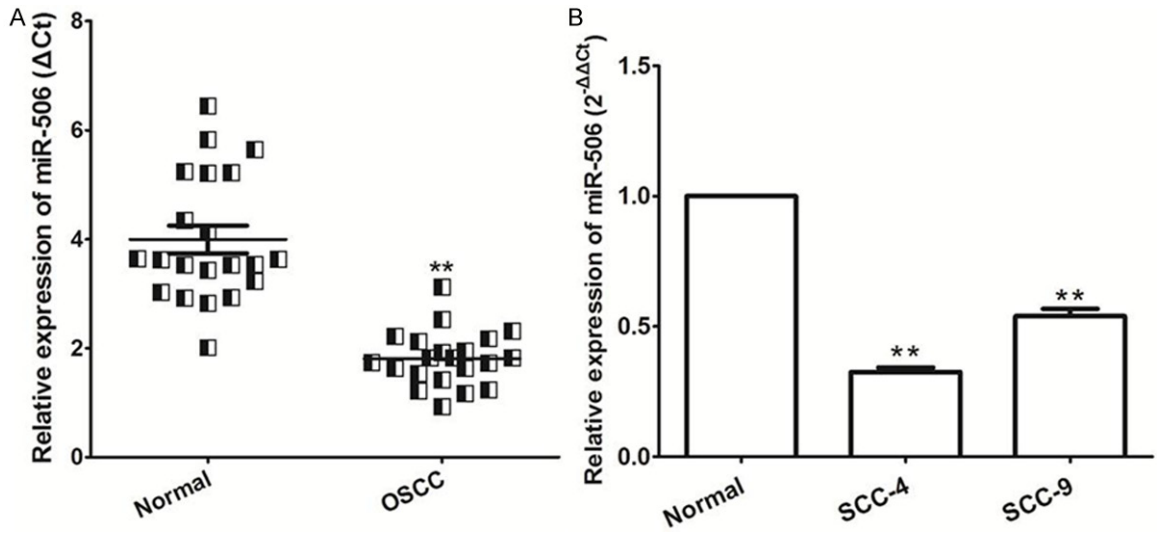

Figure 1. miR-506 expression is decreased in OSCC tissue samples. A. Expression level of miR-506 in OSCC tissues and adjacent non-cancerous tissues ($\Delta Ct = (Ct_{miR\text{-}506} - Ct_{U6})$). B. Expression level of miR-506 in SCC-4 and SCC-9 cells and normal oral mucosa cells ($\Delta\Delta Ct = (Ct_{miR\text{-}506} - Ct_{U6})_{oscc} - (Ct_{miR\text{-}506} - Ct_{U6})_{normal}$). Data represented the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with the normal group.

overexpression plasmid was obtained from Shanghai SBO Medical Biotechnology CO., LTD. The transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol.

Cell proliferation assay

The MTT assay was used to measure cell proliferation. The cells were seeded into 96-well plates at a density of 4000 cells/well. An MTT solution (20 ul of 5 mg/ml) was added to the medium and incubated for 4 h at 37°C after 24, 48, 72 h of transfection. Then the supernatant was discarded, and 150 ul of DMSO was added to each well. Optical density (OD) was detected at the wavelength of 492 nm. All experiments were performed in triplicate.

Colony formation assay

The cells were seeded in 12-well plates at 3000 cells/well after 24 h of transfection. After incubation at 37°C under 5% CO_2 for 2 weeks, the colonies were fixed with 75% ethanol for 10 min, dried and stained with 0.1% crystal violet solution for 10 min. Then the colonies were taken pictures and counted under a microscope.

Luciferase reporter assay

HEK293 cells were seeded in 96-well plates (4000 cells per well) and cells were co-transfected with 100 ng of pre-miR-506 and 100 ng of GTAT6-Wt or GTAT6-Mut. Firefly and renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega) at 24 h after transfection, and luciferase activity was normalized with renilla luciferase activity. Each assay was repeated in triplicate.

Western blotting

Cultured cells were lysed in RIPA buffer (Sigma Aldrich). Total protein was separated by 8% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membrane was incubated with rabbit antibody against human GATA6 (Cell Signaling Technology), followed by secondary antibody labeled with HRP and detected by ECL. Protein quantity was detected by β-actin as loading control.

Migration and invasion assays

The migration and invasion assays were measured with uncoated (for migration) or coated Matrigel (for invasion). After 48 h transfection, cells were added to the upper chamber of transwell inserts (BD Biocoat Matrigel 24-well invasion chamber) in medium containing 0.1% FBS, and the number of cells that invaded into the lower chamber were stained with 0.1% crystal violet solution and counted after 24 h of incubation at 37°C under 5% CO_2.

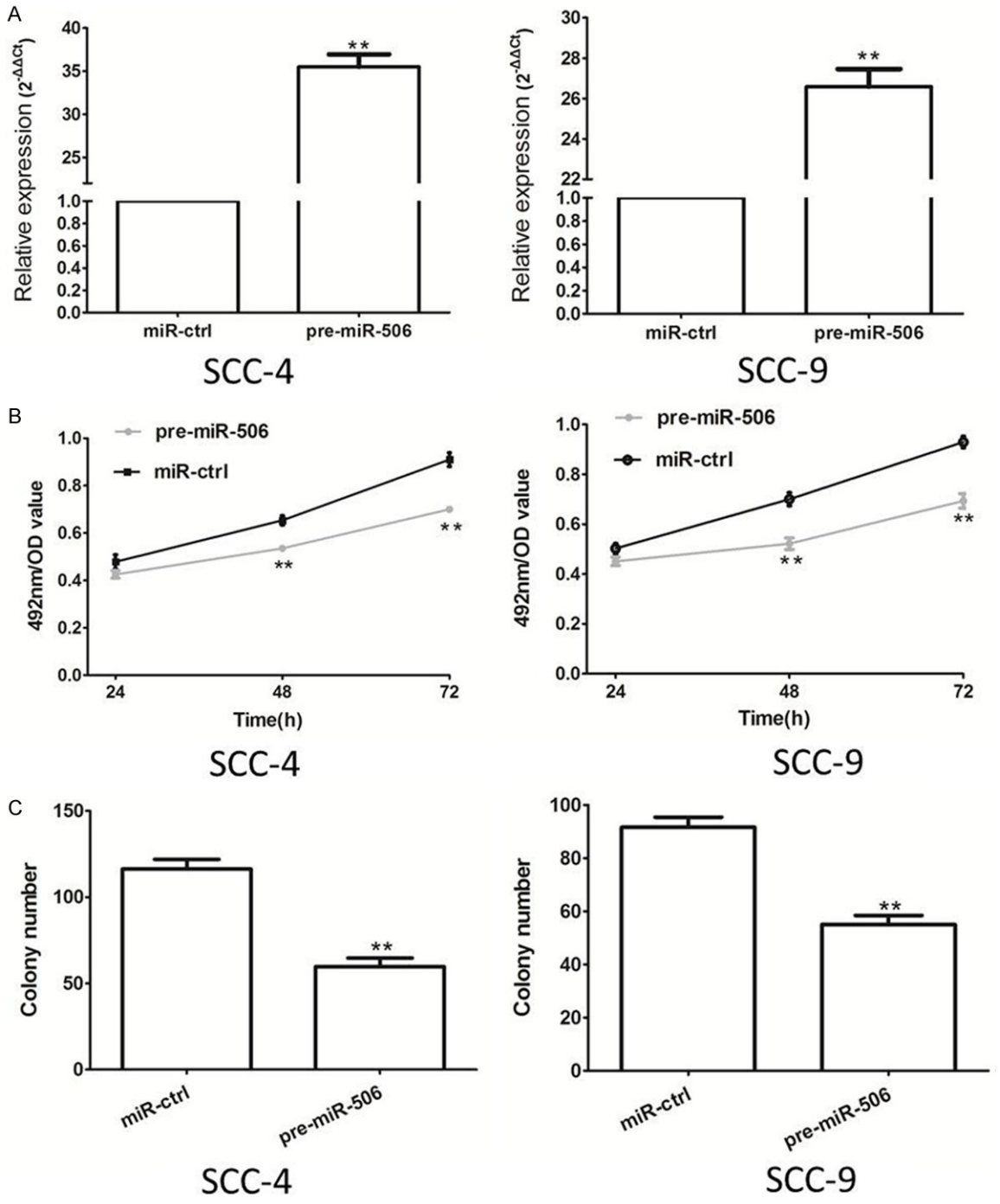

Figure 2. miR-506 inhibits the growth of OSCC cells. (A) Expression of miR-506 in SCC-4 and SCC-9 cells transfected with pre-miR-506 or miR-ctrl ($\Delta\Delta Ct = (Ct_{miR\text{-}506} - Ct_{U6})_{oscc} - (Ct_{miR\text{-}506} - Ct_{U6})_{miR\text{-}ctrl}$). MTT assay (B) and colony formation assay (C) in SCC-4 and SCC-9 cells transfected with pre-miR-506 or miR-ctrl (OD value presents absorbance value). Data represented the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with the miR-ctrl group.

Statistical analysis

Statistical analysis was performed using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SEM. The differences between groups were analyzed using *t* test. The differences were considered statistically significant when *P < 0.05, **P < 0.01.

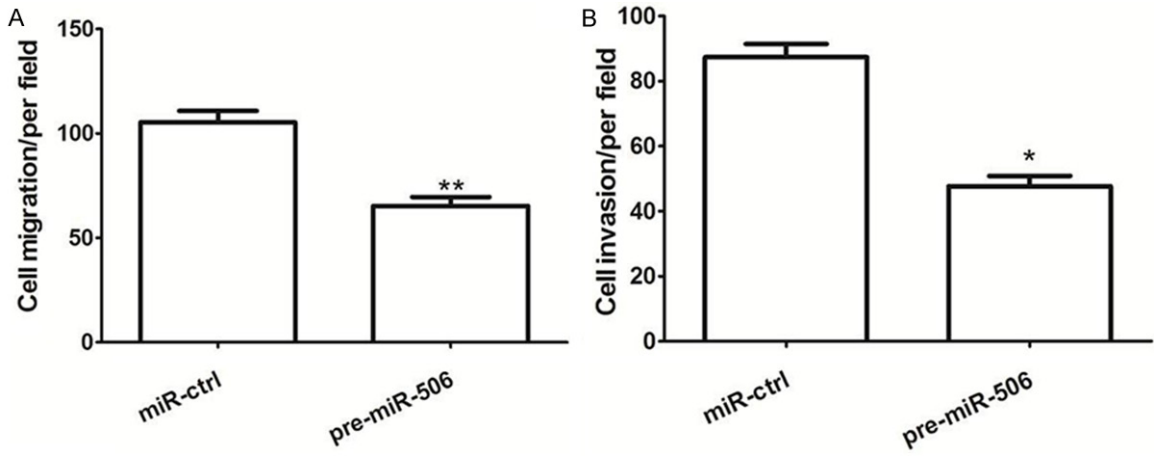

Figure 3. miR-506 suppresses metastasis of OSCC cells. Migration assay (A) and invasion assay (B) in SCC-4 and SCC-9 cells transfected with pre-miR-506 or miR-ctrl, crystal violet-stained cells in 4 random fields were counted. Data represented the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with the miR-ctrl group.

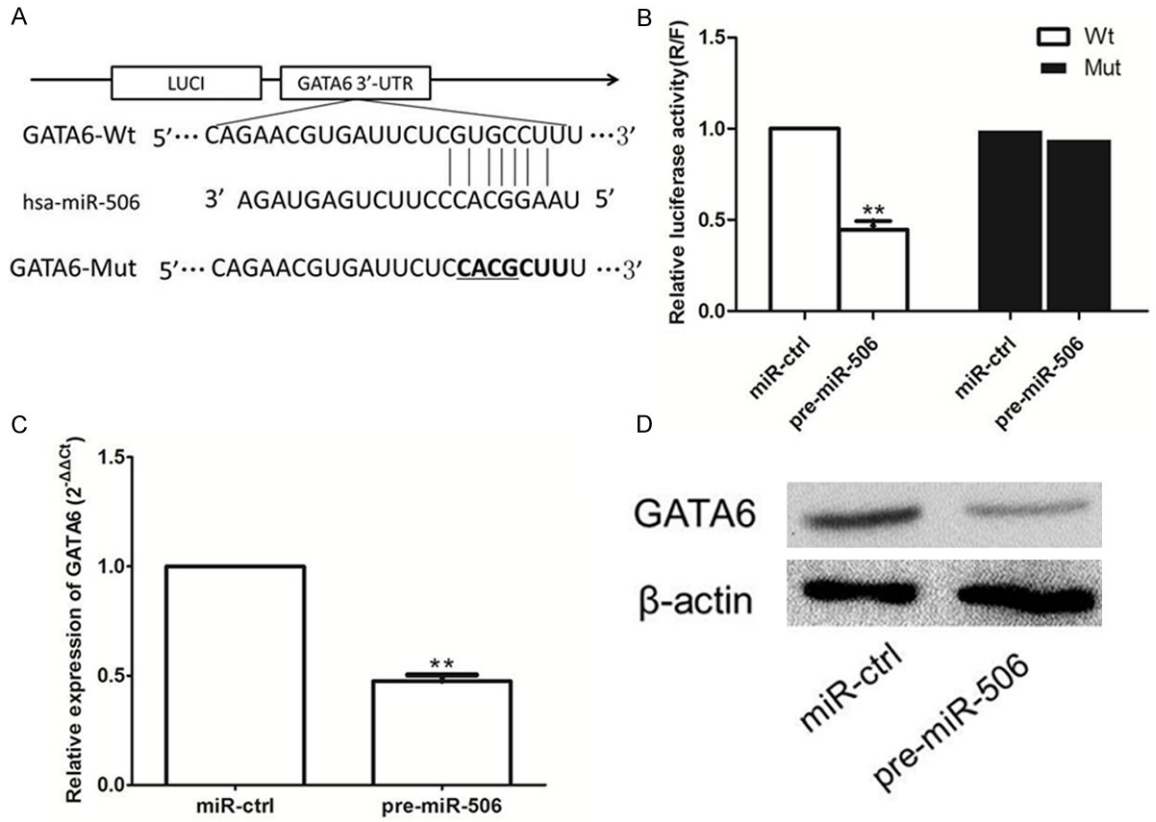

Figure 4. GATA6 is a direct target of miR-506. A. The potential miR-506 targeting site in GATA6 3'-UTR and the mutated sequence. B. Luciferase activity was detected when HEK-293 cells were co-transfected with pre-miR-506 with Wt or Mut of GATA6 3'-UTR. C. Expression of GATA6 mRNA was measured by qRT-PCR in SCC-4 cells transfected with pre-miR-506 or miR-ctrl. D. GATA6 protein level was detected by western blotting in SCC-4 cells transfected with pre-miR-506 or miR-ctrl. Data represented the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with the miR-ctrl group.

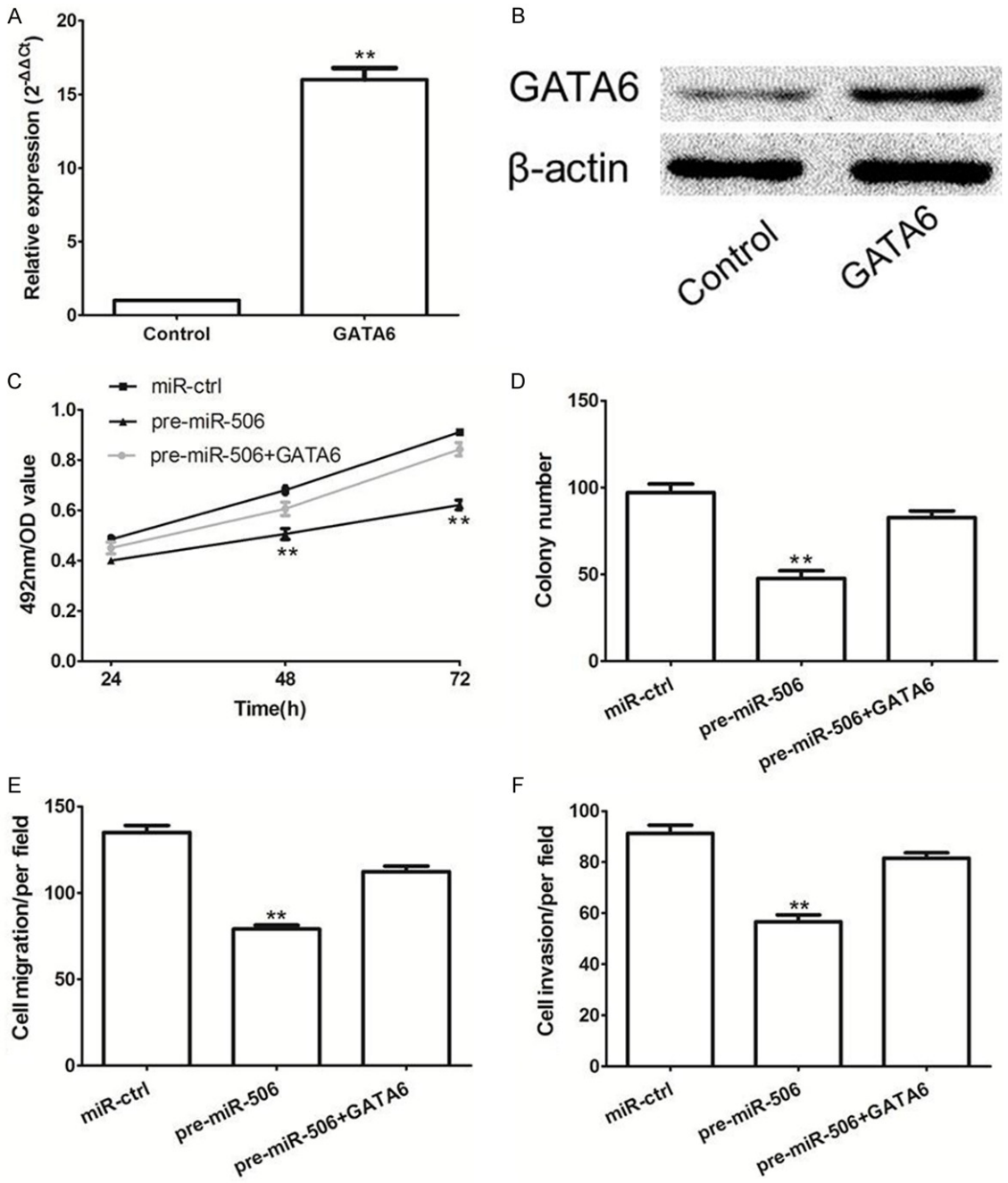

Figure 5. GATA6 overexpression attenuates the tumor suppressive effects of miR-506. A. Expression of GATA6 mRNA was measured by qRT-PCR in SCC-4 cells transfected with GATA6 overexpression plasmid. B. GATA6 protein level was detected by western blotting in SCC-4 cells transfected with GATA6 plasmid. C-F. MTT, colony formation, migration and invasion were performed when SCC-4 cells were transfected with miR-506 with/without GATA6 overexpression plasmid. Data represented the mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with the miR-ctrl group.

Results

miR-506 expression is decreased in OSCC tissue samples and cell lines

qRT-PCR was used to measure the expression of miR-506 in 21 paired OSCC tissue samples (**Table 1**). As shown in **Figure 1A**, we found that the relative expression level of miR-506 was significantly lower in OSCC tissues compared with the adjacent normal tissues (P < 0.001). miR-506 was also decreased in SCC-4 and SCC-9 cells compared with normal oral mucosa cells (**Figure 1B**, P < 0.001).

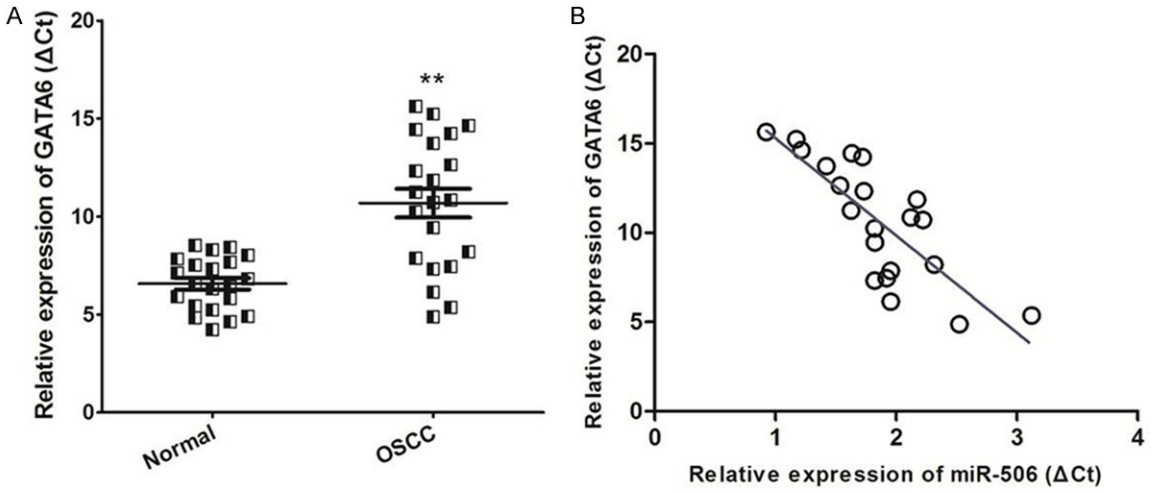

Figure 6. miR-506 expression is negatively correlated with GATA6 in OSCC tissues. A. Expression of GATA6 mRNA was measured by qRT-PCR in OSCC tissues and adjacent non-cancerous tissues. Data represented the mean ± SEM from three independent experiments. **P < 0.01 compared with the normal tissues. B. GATA6 mRNA level was inversely correlated with miR-506 level in OSCC tissues (ΔCt = ($Ct_{miR-506}$ - Ct_{U6}) for miR-506; (Ct_{GATA6} - $Ct_{\beta-actin}$)$_{oscc}$ - (Ct_{GATA6} - $Ct_{\beta-actin}$)$_{normal}$ for GATA6).

miR-506 suppresses growth of OSCC cells

We investigated the effect of miR-506 on the growth of OSCC cells using MTT and colony formation. The expression of miR-506 was identified by qRT-PCR in OSCC cells transfected with pre-miR-506 (**Figure 2A**, P < 0.001, P = 0.001, respectively). As shown in **Figure 2B**, overexpression of miR-506 significantly inhibited proliferation of SCC-4 and SCC-9 cells compared with the empty vector (miR-ctrl) group (P < 0.05). Similarly, miR-506 overexpression significantly suppressed colony formation of SCC-4 and SCC-9 cells compared with the miR-ctrl group (**Figure 2C**, P = 0.002, P = 0.002, respectively).

miR-506 inhibits migration and invasion of OSCC cells

As shown in **Figure 1B**, the expression of miR-506 was significantly lower in SCC-4 cells than SCC-9 cells. To identify the role of miR-506 for the metastasis of OSCC cells, pre-miR-506 or miR-ctrl was transfected into SCC-4 cells and then migration and invasion assays were performed. As shown in **Figure 3A** and **3B**, miR-506 overexpression significantly decreased the migration and invasion abilities of SCC-4 cells compared with miR-ctrl group (P = 0.004, P = 0.002, respectively).

GATA6 is a direct target of miR-506

GATA6 was selected as the potential target of miR-506 using TargetScan 6.2 (http://www.targetscan.org/) depending on predicted consequential pairing of target region and miRNA, seed match and context score percentile etc., Wild type (Wt) or the mutated (Mut) of GATA6 3'-UTR sequence was cloned into the luciferase reporter vector (**Figure 4A**). Then we found that miR-506 overexpression remarkably suppressed the luciferase activity of the GATA6-Wt 3'-UTR, without effect on GATA6-Mut 3'-UTR in HEK-293 cells (**Figure 4B**, P < 0.001). In addition, the mRNA and protein levels of GATA6 were decreased in SCC-4 cells transfected with pre-miR-506 compared with miR-ctrl group (**Figure 4C** and **4D**).

miR-506 suppresses OSCC progression by targeting GATA6

We further investigated that whether overexpression of GATA6 could attenuate the effects of miR-506 on OSCC cells using MTT, colony formation, migration and invasion. The GATA6 overexpression was validated by qRT-PCR and western blotting analyses (**Figure 5A** and **5B**). As shown in **Figure 5C-F**, GATA6 overexpression significantly attenuated the tumor suppressive effects of miR-506 on SCC-4 cells, including

proliferation, colony formation, migration and invasion abilities.

miR-506 expression is inversely correlated with GATA6 expression in OSCC tissues

Finally, we examined the expression of GATA6 in 21 paired OSCC tissues using qRT-PCR. We demonstrated that GATA6 mRNA was remarkably increased in OSCC tissues compared with the adjacent normal tissues (**Figure 6A**, $P < 0.001$). Furthermore, as shown in **Figure 6B**, miR-506 expression was inversely correlated with GATA6 mRNA expression in OSCC tissues ($r = -0.814$, $P < 0.001$).

Discussion

In present study, our results demonstrated that miR-506 could act as a potential tumor suppressor in OSCC. Furthermore, GATA6 was identified as a direct target of miR-506 in OSCC cells, and the tumor suppressive effects of miR-506 could be completely restored by GATA6 overexpression. In addition, we also found that GATA6 was inversely correlated with miR-506 in OSCC tissues.

miR-506 was an tumor suppressive miRNA and downregulated in various types of cancers. Aberrant expression of miR-506 was reported to contribute to the malignant phenotype of several tumors, including hepatocellular carcinoma, cervical cancer and ovarian cancer [11, 13, 14]. miR-506 expression was downregulated in 80% of the cervical cancer samples. miR-506 induced cell cycle arrest at the G1/S transition, and promoted apoptosis and chemosensitivity of cervical cancer cell through targeting Gli3 [13]. Liu et al. [11] shown that overexpression of miR-506 could activate FOXM1 signaling to inhibit proliferation and promote senescence of ovarian cancer cells by targeting CDK4 and CDK6. Moreover, in breast cancer, miR-506 overexpression inhibited TGFβ-induced epithelial-mesenchymal transition and suppressed adhesion, invasion and migration of MDA-MB-231 human breast cancer cells [12]. From above studies, we inferred that miR-506 might act as a tumor suppressor, but the role of miR-506 in OSCC is unknown. In our study, the low expression of miR-506 was firstly identified in OSCC tissues and cell lines. Furthermore, we found that the overexpressed of miR-506 could inhibit proliferation, migration and invasion of OSCC cells.

GATA6, a member of the GATA family of zinc-finger transcription factors, is the earliest marker of the primitive endoderm lineages. Cumulative reports indicated that the expression of GATA6 was increased in several cancers [15-17]. Whissell et al. [18] indicated that GATA6 competed with β-catenin/TCF4 for binding to a distal regulatory region of the BMP4 locus, which increased susceptibility to development of colorectal cancer. RNA interference against GATA6 reduced the levels of the latter transcripts and arrested cells in G2 and M phases of the cell cycle and inhibited cell proliferation and increased apoptosis in pancreatic cancer and gastric cancer cells [15, 17]. In addition, Tsuji et al. [19] reported that GATA6 was upregulated in colorectal cancer cells due to the downregulation of miR-363, then promoted the tumorigenicity of colorectal cancer cells. However, the expression and function of GATA6 is unclear in tumorigenesis of OSCC. In our study, GATA6 was identified as a direct target of miR-506 in OSCC cells and GATA6 overexpression significantly attenuated the tumor suppressive effects of miR-506 on OSCC cells. Moreover, we also demonstrated that GATA6 was inversely correlated with miR-506 in OSCC tissues.

In conclusion, our study is the first to report the tumor suppressor roles of miR-506 in the progression of OSCC. miR-506 overexpression can suppress growth and metastasis of OSCC cells by targeting the expression of GATA6. These findings indicate that the restoration of the tumor suppressor miR-506 might be a potential candidate for the treatment of OSCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Hui Liu, Department of Stomatology, Jining NO. 1 People's Hospital, NO. 6, Jiankang Road, Jining City 272011, Shandong Pro-

vince, P. R. China. Fax: +86 0537-2253417; E-mail: liuhui_270000@163.com

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