Abstract. MicroRNAs (miRNAs) play a pivotal role in cancer progression and development, representing novel therapeutic tools for cancer therapy. Forkhead box Q1 (FOXQ1) functions as an oncogene in various cancer types. However, the functional significance of FOXQ1 in cervical cancer remains unknown. In this study, we investigated the biological function of FOXQ1 in cervical cancer and tested whether or not FOXQ1 can be targeted and regulated by specific miRNAs. We found that FOXQ1 was highly expressed in cervical cancer cell lines. Knockdown of FOXQ1 by small interfering RNA (siRNA) significantly suppressed the proliferation and epithelial-mesenchymal transition (EMT) of cervical cancer cells. FOXQ1 was predicted as a target gene of microRNA-506 (miR-506), and this prediction was validated by dual-luciferase reporter assay. Quantitative real-time PCR and western blot analyses demonstrated that mRNA and protein expression was negatively regulated by miR-506. The expression of miR-506 was downregulated in cervical cancer tissues, and miR-506 expression was inversely correlated with FOXQ1 expression in cervical cancer. The overexpression of miR-506 dramatically suppressed the proliferation and EMT of cervical cancer cells that mimicked the suppression of FOXQ1 siRNA. Furthermore, the restoration of FOXQ1 expression significantly reversed the inhibitory effect of miR-506. Overall, our study demonstrated that miR-506 inhibited the proliferation and EMT of cervical cancer cells by targeting FOXQ1 and provided evidence that the miR-506/FOXQ1 axis plays an important role in the pathogenesis of cervical cancer, representing potential molecular targets for the development of anticancer agents for cervical cancer treatment.

Introduction

Cervical cancer is a common cancer type in women that severely affects women's health worldwide (1). Although cancer treatments have been improved in recent decades, the treatment outcomes of cervical cancer patients have not been significantly impaired and cervical cancer remains the second leading cause of cancer-related death in women (2). Although much research has focused on cervical cancer, the precise molecular mechanisms underlying the pathogenesis of this disease remain poorly understood. Thus, finding new targets for the development of effective therapeutics for cervical cancer is important.

Forkhead box (FOX) proteins are a family of transcriptional regulators that are associated with regulating various cellular processes, including cell proliferation and survival, apoptosis, differentiation and metabolism (3). Thus, FOX proteins contribute to the development and progression of cancers (4). Among the FOX family members, forkhead box Q1 (FOXQ1) is reportedly associated with various types of cancers. FOXQ1 regulates the proliferation, migration, invasion, metastasis, epithelial-mesenchymal transition (EMT), and chemosensitivity of cancer cells by regulating gene networks (5-9). The increased EMT of cancer cells accounts for cancer metastasis by promoting the migratory and invasive abilities of cancer cells (10,11). FOXQ1 has been suggested as an oncogene in various cancers, including colorectal (12), lung (13,14), gastric (15), ovarian (16) and breast cancers (17). However, whether or not FOXQ1 plays an important role in cervical cancer remains poorly understood.

The role of microRNAs (miRNAs) in tumorigenesis has been widely studied in recent decades (18). miRNAs negatively regulate gene expression by directly targeting the 3’-untranslated region (UTR) of mRNAs, leading to translation inhibition (19). miRNAs modulate numerous cancer cell
processes, including proliferation, apoptosis, migration and invasion, by regulating target genes (20). Thus, miRNAs are promising targets for developing anticancer drugs. A previous study suggested that miRNAs can be used as biomarkers or therapeutic targets for the diagnosis or treatment of cervical cancer (21). Various studies have demonstrated that miRNAs such as miR-21 (22) and miR-1246 (23) are dysregulated in cervical cancer. Some miRNAs could inhibit cervical cancer cell growth by suppressing target genes (24,25). However, the precise role of miRNAs in cervical cancer pathogenesis remains unclear.

In this study, we investigated the role of FOXQ1 in cervical cancer and tested the potential of specific miRNAs to inhibit FOXQ1 expression. FOXQ1 was overexpressed in cervical cancer cell lines, and knockdown of FOXQ1 by small interfering RNA (siRNA) significantly inhibited the proliferation and EMT of the cervical cancer cells. Notably, FOXQ1 was indicated as the target gene of miR-506. The overexpression of miR-506 significantly suppressed FOXQ1 expression and inhibited the proliferation and EMT of cervical cancer cells. Furthermore, restoration of FOXQ1 expression partially reversed the suppression of miR-506. Overall, our study demonstrated that miR-506 targets FOXQ1 to inhibit the proliferation and EMT of cervical cancer cells.

Materials and methods

Cell lines and clinical specimens. The human normal skin keratinocyte line HaCaT and the human cervical cancer cell lines CaSki and SiHa were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the human cervical cancer cell lines Caski and SiHa were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and negative control miRNA mimics (miR-NC; 5'-UUCUCCGAACGUUCACGUTT-3'), miR-506 mimics (5'-UAAGGCACCCUUCCUGAUGA-3') and negative control miRNA mimics (miR-NC; 5'-UUCUCCGAAACGUGUCAGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-506 mimics were transfected into cells at final concentrations of 50 nM using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. For knockdown of FOXQ1, ~1 µg of FOXQ1 siRNA was diluted into 100 µl of DMEM containing 6 µl of Lipofectamine 2000 and incubated for 45 min at room temperature. The siRNA transfection reagent mixture was added to each well and incubated for 7 h. Then, the medium was replaced with fresh medium and cultured for 48 h. The open reading frame of FOXQ1 without 3'-UTR was inserted into pcDNA3.0 plasmids. For the rescue experiment, miR-506 mimics (50 nm) and pcDNA3/FOXQ1 overexpression vectors (3 µg) were co-transfected into cervical cancer cells for 48 h. Empty vectors were used as control.

Cell proliferation assay. Cells were grown into 96-well plates (1x10^4 cells/well), transfected with FOXQ1 siRNA or miR-506 mimics, and then incubated for 24, 48, and 72 h. Cell proliferation was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. In brief, 20 µl of MTT solution (5 mg/ml; Sigma) was added to each well after replacement with fresh medium. After 4 h, the formazan crystals were melted by adding 200 µl of dimethyl sulfoxide (DMSO) to each well. After the crystals

Western blot analysis. Proteins were extracted from cells using RIPA lysis buffer containing protease inhibitor cocktail (Beyotime, Haimen, China), and the concentration was measured and quantified using the Bradford method. A total of 25 µg proteins from each cell group were separated via 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins on the gel were transferred onto a nitrocellulose membrane (Millipore, Boston, MA, USA), which was then blocked by blocking solution (3% nonfat-dried milk) for 1 h at 37°C. After blocking, the membrane was incubated with primary antibodies, including anti-FOXQ1 (1:400) and anti-GAPDH (1:10,000) (both from Abcam, Cambridge, UK), which were diluted in blocking buffer at 4°C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; Beyotime, Shanghai, China) for 1 h at 37°C. The protein bands were detected using chemiluminescence (Amersham, Little Chalfont, UK). Densitometric analysis of the protein bands was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Cell treatment. The expression of FOXQ1 was suppressed by FOXQ1 siRNA or miR-506 mimic transfection. FOXQ1 siRNA (5'-CCCGGACUUUGCACUUUGA-3') and negative control siRNA (NC-siRNA; 5'-UUCUCCGAACGUUCACGCU-3'), miR-506 mimics (5'-UAAGGCACCCUUCCUGAUGA-3') and negative control miRNA mimics (miR-NC; 5'-UUCUCCGAAACGUGUCAGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-506 mimics were transfected into cells at final concentrations of 50 nM using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. For knockdown of FOXQ1, ~1 µg of FOXQ1 siRNA was diluted into 100 µl of DMEM containing 6 µl of Lipofectamine 2000 and incubated for 45 min at room temperature. The siRNA transfection reagent mixture was added to each well and incubated for 7 h. Then, the medium was replaced with fresh medium and cultured for 48 h. The open reading frame of FOXQ1 without 3'-UTR was inserted into pcDNA3.0 plasmids. For the rescue experiment, miR-506 mimics (50 nm) and pcDNA3/FOXQ1 overexpression vectors (3 µg) were co-transfected into cervical cancer cells for 48 h. Empty vectors were used as control.
were dissolved, the optical density of each well was measured at 490 nm using an ELISA reader.

**Luciferase reporter assay.** The cDNA fragments of FOXQ1 3'-UTR containing the predicted binding site for miR-506 were inserted into pmirGLO luciferase promoter vectors (Promega, Madison, WI, USA). The cells were seeded into 24-well plates (5x10^4 cells/well) and were then transfected with 500 ng of pmirGLO-FOXQ1 3'-UTR and 50 nM of miR-506 mimics for 48 h using Lipofectamine 2000. The cells were lysed, and the activity of firefly or Renilla luciferase was detected using a dual-luciferase reporter assay kit (Promega) in accordance with the manufacturer's instructions.

**Statistical analysis.** Data are shown as means ± standard deviation. SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Differences between two groups were analyzed using the Student's t-test. One-way ANOVA was conducted in multiple groups for statistical analyses. The correlation between FOXQ1 and miR-506 was evaluated using Spearman's correlation analysis. Statistical significance was considered at P<0.05.

**Results**

**High expression of FOXQ1 is detected in cervical cancer cells.** To explore the potential role of FOXQ1 in cervical cancer, we detected FOXQ1 expression in the cervical cancer cell lines CaSki and SiHa through qRT-PCR and Western blot analyses. qRT-PCR data showed that the mRNA expression of FOXQ1 was significantly higher in the CaSki and SiHa cells than that in the HaCaT cells (Fig. 1A). Western blot analysis also revealed a high protein expression level of FOXQ1 in the CaSki and SiHa cells (Fig. 1B). The data indicated that FOXQ1 may function as an oncogene in cervical cancer.

**Knockdown of FOXQ1 by siRNA suppresses cervical cancer cell proliferation.** To understand the biological role of FOXQ1 in cervical cancer, we performed loss-of-function experiments in the CaSki and SiHa cells by using siRNA targeting FOXQ1. Cells were transfected with FOXQ1 siRNA, and the expression of FOXQ1 was detected by qRT-PCR and western blot analyses. The mRNA and protein expression levels of FOXQ1 were significantly decreased by FOXQ1 siRNA transfection (Fig. 2A and B). Furthermore, the effect of FOXQ1 silencing on cell proliferation was detected by MTT assay. Transfection of FOXQ1 siRNA markedly impeded the proliferation of CaSki (Fig. 2C) and SiHa (Fig. 2D) cells. These results imply that FOXQ1 is a potential molecular target for inhibiting the proliferation of cervical cancer cells.

**Knockdown of FOXQ1 by siRNA inhibits the EMT of cervical cancer cells.** To further investigate the function of FOXQ1, we determined the effect of FOXQ1 silencing on the EMT of cervical cancer cells. Results showed that FOXQ1 knockdown significantly increased the expression of the epithelial marker E-cadherin and decreased the expression of the mesenchymal marker vimentin in the CaSki (Fig. 3A) and SiHa (Fig. 3B) cells transfected with FOXQ1 siRNA. The data suggest that FOXQ1 may be a potential target for inhibiting cervical cancer metastasis.

**FOXQ1 is a potential target of miR-506.** Previous studies have reported that FOXQ1 expression can be regulated by specific miRNAs in cancers (26,27). Bioinformatic research revealed that FOXQ1 has a putative binding site for miR-506 (Fig. 4A), a well-recognized tumor-suppressor miRNA (28,29). A dual-luciferase reporter assay was performed to verify whether or not FOXQ1 is a target gene of miR-506. The results of the luciferase assay showed that miR-506 overexpression significantly inhibited the luciferase activity of the reporter gene with the wild-type FOXQ1 3'-UTR but not with the mutant FOXQ1 3'-UTR in the CaSki (Fig. 4B) and SiHa cells (Fig. 4C). These results indicate that FOXQ1 is a direct target gene of miR-506.

**miR-506 negatively regulates FOXQ1 expression.** To further confirm that FOXQ1 is the target gene of miR-506, we detected the expression change of FOXQ1 in response to miR-506 mimic transfection. The overexpression of miR-506 through the transfection of miR-506 mimics significantly suppressed the mRNA expression of FOXQ1, confirming FOXQ1 as a target of miR-506.
expression of FOXQ1 in the cervical cancer cell lines (Fig. 5A). Furthermore, the protein expression of FOXQ1 was also significantly decreased by miR-506 overexpression (Fig. 5B).

To further investigate the relationship between miR-506 and FOXQ1, we examined the expression levels of miR-506 and FOXQ1 in clinical specimens and analyzed their correlation. The expression of miR-506 (Fig. 6A) was significantly downregulated in the cervical cancer tissues, whereas that of FOXQ1 (Fig. 6B) was significantly upregulated in the cervical cancer tissues when compared with the adjacent non-tumor tissues. Furthermore, correlation analysis revealed a marked inverse correlation between FOXQ1 and miR-506 expression in the cervical cancer tissues (Fig. 6C). Overall, these results indicate that miR-506 negatively regulates the expression of FOXQ1, and the high expression of FOXQ1 in cervical cancer may be caused by the dysregulation of miR-506.
Overexpression of miR-506 inhibits the proliferation and EMT of cervical cancer cells. Considering the regulatory function of miR-506 on FOXQ1 expression as described above, we speculated that miR-506 plays an important role in cervical cancer. To test this hypothesis, we detected the effect of miR-506 overexpression on cervical cancer cell proliferation. MTT assay showed that the proliferation of the cervical cancer CaSki (Fig. 7A) and SiHa (Fig. 7B) cells transfected with miR-506 mimics was significantly decreased. Furthermore, the transfection of miR-506 mimics significantly increased the expression of E-cadherin and decreased the expression of vimentin in the CaSki (Fig. 7C) and SiHa (Fig. 7D). These results revealed that miR-506 inhibited the proliferation and EMT of cervical cancer cells.

miR-506 suppresses the proliferation and EMT of cervical cancer cells through FOXQ1. To validate whether miR-506 exerts its tumor-suppressive function by suppressing FOXQ1, we performed rescue experiments. Results showed that the restoration of FOXQ1 expression (Fig. 8A) by transfecting pcDNA3/FOXQ1 overexpression vectors partially rescued the inhibitory effect of miR-506 overexpression on the proliferation (Fig. 8B) and EMT (Fig. 8C and D) in CaSki cells. Similar results were obtained in the SiHa cells (data not shown). Thus, these results revealed that miR-506 functions through FOXQ1 in cervical cancer.

Discussion

The present study was the first to report that FOXQ1 regulates the proliferation and EMT of cervical cancer cells. Moreover, miR-506 directly targeted and regulated FOXQ1 expression, representing a novel tool for the treatment of cervical cancer by targeting FOXQ1.

The FOXQ1 gene, which encodes a protein of 403 amino acids, is predominantly expressed in the bladder, salivary...
FOXQ1 is overexpressed in colorectal and lung cancer cell lines (13). FOXQ1 was later found to be an oncogene in various types of cancer. Kaneda et al (12) reported that FOXQ1 expression is increased in colorectal cancer tissues and promotes tumorigenicity and tumor growth in vitro and in vivo. High FOXQ1 expression is tightly linked to the aggressive malignant phenotype of hepatocellular carcinoma (30). FOXQ1 expression is significantly upregulated in gastric cancer tissues and is positively related to tumor size, histological grade, lymph node metastasis, and poor prognosis of gastric cancer (15). Furthermore, high expression of FOXQ1 promotes the proliferation and metastasis of glioma (7) and esophageal cancer cells (5). However, the role of FOXQ1 in cervical cancer is poorly understood. In
the present study, we found that the mRNA and protein levels of FOXQ1 were overexpressed in cervical cancer cell lines. This study is the first to report the role of FOXQ1 in cervical cancer. Knockdown of FOXQ1 inhibited the proliferation of cervical cancer cells. These results further indicate that FOXQ1 is an oncogene, which may be a promising molecular target for cervical cancer.

EMT is a critical step in cancer metastasis (10,11). Increasing studies have demonstrated that FOXQ1 plays an important role in regulating EMT. FOXQ1 promotes the EMT of mammary epithelial cells and breast cancer metastasis (17). FOXQ1 induces EMT by inhibiting the epithelial regulator E-cadherin in human cancers (8). High FOXQ1 expression in lung cancer tissues correlates with the loss of E-cadherin (14). Fan et al (31) reported that FOXQ1 protein interacts with the promoter region of E-cadherin and promotes the EMT of mammary epithelial cells. Knockdown of FOXQ1 suppresses invasion and metastasis by the reversal of EMT in bladder cancer (32). Consistent with these findings, the present results demonstrated that FOXQ1 regulated the EMT of cervical cancer cells. We found that FOXQ1 knockdown increased the expression of the epithelial marker E-cadherin and decreased the expression of the mesenchymal marker vimentin, implying the EMT suppression of cervical cancer. These reports and our findings support the notion that FOXQ1 is a potential molecular target for inhibiting EMT and the metastasis of cervical cancer.

Inhibiting oncogene expression by specific miRNAs could be a novel strategy for the treatment of cancer. To date, the miRNAs that specifically target and inhibit FOXQ1 have not been well recognized. In the present study, miR-506 directly targeted the 3'-UTR of FOXQ1 and inhibited the mRNA and protein expression of FOXQ1. Further analysis showed that miR-506 expression was decreased in the cervical cancer tissues and was inversely correlated with the increased expression of FOXQ1. Thus, the overexpression of miR-506 inhibited the proliferation and EMT of cervical cancer cells. Moreover, the restoration of FOXQ1 expression significantly reversed the inhibitory effect of miR-506. These data indicate that miR-506 suppresses the proliferation and EMT of cervical cancer cells by targeting FOXQ1. Our findings agree with a recent study that reported that miR-506 suppresses the proliferation and invasion of nasopharyngeal cancer cells by targeting and inhibiting FOXQ1 (33). Overall, miR-506 may be a promising miRNA for targeting FOXQ1. In addition, miR-124 is reportedly capable of targeting and inhibiting FOXQ1 to suppress tumor growth and metastasis in nasopharyngeal cancer (27). Zhang et al (26) reported that FOXQ1 is a target gene of miR-422a and that the overexpression of miR-422a inhibited the proliferation and migration of hepatocellular carcinoma. miR-1271 targets FOXQ1 to suppress the proliferation and EMT of gastric cancer cells (34). These findings further support that targeting FOXQ1 by using specific miRNAs could be a potential strategy for cancer therapy.

In this study, miR-506 inhibited the proliferation and EMT of cervical cancer cells by targeting FOXQ1. We further demonstrated that miR-506 expression was downregulated in cervical cancer tissues. miR-506 functioned as a tumor-
suppressive miRNA. In fact, increasing evidence supports the tumour-suppressive role of miR-506 in various cancer types. The overexpression of miR-506 inhibited the proliferation, metastasis, and EMT of breast cancer cells (35,36). Furthermore, miR-506 suppressed the metastasis of breast cancer cells by targeting the IQ motif containing GTPase-activating protein 1 (29). In gastric cancer, miR-506 inhibited EMT by targeting ETS1 (37) or SNAI2 (38). miR-506 also suppressed the proliferation and invasion of gastric cancer cells by targeting Yap1 (39). Furthermore, miR-506 served a tumour-suppressive function in hepatocellular carcinoma (40), colon cancer (28), and oral squamous cell carcinoma (41) by targeting various oncogenes. In the present study, we demonstrated the critical role of miR-506 in cervical cancer. Our results were consistent with those of a recent study that reported that miR-506 is significantly downregulated in cervical cancer tissues (42). These authors further demonstrated that miR-506 overexpression suppressed the growth and enhanced apoptosis and chemosensitivity of cervical cancer cells by targeting Gli3 (42). Overall, decreased miR-506 expression may result in the overexpression of oncoproteins, such as FOXQ1, which contributes to the development and progression of cervical cancer.

In conclusion, our study elucidated that FOXQ1 functions as an oncogene and is related to the proliferation and EMT of cervical cancer cells. FOXQ1 could be targeted and inhibited by miR-506, leading to the suppression of the proliferation and EMT of cervical cancer cells. These results suggest that the miR-506/FOXQ1 axis plays an important role in the pathogenesis of cervical cancer, representing potential molecular targets for the development of anticancer agents.

References


