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Effect of miR-212 targeting TCF7L2 on the proliferation and metastasis of cervical cancer

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Abstract. – OBJECTIVE: MicroRNAs (miRs) function as either oncogenes or tumor suppressors in the progression of various human cancers, including cervical cancer. This study aimed to explore the role of miR-212 in cervical cancer and the mechanisms underlying this role.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (RT-PCR) and Western blot assays were used to determine the expression levels of miR-212 and TCF7L2 in the cervical cancer cells. Cell proliferation invasion was examined using BrdU assays and transwell, respectively. A bioinformatics analysis was used to predict targets, and a dual-luciferase reporter system was applied for validation.

RESULTS: In our study, we demonstrated that miR-212 expression was significantly downregulated in cervical cancer tissues and cell lines. Moreover, the increased expression of miR-212 suppressed cell proliferation and invasion of cervical cancer cell lines *in vitro*. On the contrary, the decreased expression of miR-212 promoted cell proliferation and invasion of cervical cancer cell lines. Finally, the results of Western blot showed that overexpression of miR-212 dramatically suppressed the protein expression of TCF7L2. The knockdown of miR-212 showed the contrary effect. Luciferase reporter assay identified TCF7L2 as a novel direct target of miR-212.

CONCLUSIONS: Our results revealed that miR-212 inhibited cervical cancer metastasis and progression by targeting TCF7L2 expression.

Key Words:
miR-212, Cervical cancer, Proliferation, Invasion, TCF7L2.

Introduction

Cervical cancer is the third most common cancers worldwide, with the large majority of patients living in low-income or developing countries[1,2]. Moreover, Approximately 527,600 new cervical cancer cases and 265,700 deaths were annually reported worldwide[3]. Tumor invasion and metastasis remain formidable obstacles to the effective treatment of this disease. Thus, a better understanding of the molecular mechanisms involved in the carcinogenesis of cervical cancer would help to improve therapies for the disease.

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs approximately 18-22 nucleotides (nt) in length, and participate in the post-transcriptional regulation of gene expression by base-pairing with the 3'-UTR of target messenger RNAs (mRNAs)[4,5]. It has been known that miRNAs could influence multiple biological processes including proliferation, apoptosis, and development[6,7]. A large body of research has demonstrated that miRNAs can act as either potent oncogenes or tumor suppressor genes. For instance, Zhao et al[8] showed that miR-874 served as a tumor suppressor by targeting STAT3 in human colorectal cancer cells. Li et al[9] found that miR-522 functioned as a tumor promoter in hepatocellular carcinoma by targeting DKK1 and SFRP2. Especially, another study by Li et al[10] observed that miR-138 could inhibit proliferation of cervical cancer cells by targeting c-Met. Recently, abnormal expression of miR-212 was found in different tumors, including cervical cancer[11,12]. However, its detailed role remains largely unclear.

In this study, we observed that miR-212 was decreased in cervical cancer tissues and cell lines. We further investigate the biologi-

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cal functions and molecular mechanisms of miR-212 in human cervical cancer in vitro and to identify the target genes regulated by the miR-212.

Patients and Methods

Patients and Tissue Samples

Ten clinical cervical cancer tissues and their corresponding noncancerous tissues used in this study were obtained from Department of Obstetrics and Gynecology, Binzhou Medical University Hospital. Each surgical specimen of paired cervical cancer tumor tissues and normal adjacent tissues was instantly frozen in liquid nitrogen, and stored at -80°C until RNA extraction. The sampling process was agreed to by the patients and was performed according to the instructions of our institute under the supervision of the Ethics Committee.

Cell Culture and Transfection

Hela and Siha human cervical cancer cell lines were primarily purchased from Shanghai Institute of Biochemistry and Cell Biology (Xuhui, Shanghai, China). Cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum in a water-wormed incubator with 5% CO_2. The miR-212 mimic, miR-212 inhibitor were synthesized by RiboBio Corporation (Gaoxin, Guangdong, China), and transfected at a final concentration of 50-100 nM with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was detected by qRT-PCR after 48 h of incubation.

Quantitative RT-PCR Analysis (qRT-PCR).

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA (2 mg) using M-MLV Reverse Transcriptase Kit (Promega, Dongcheng, Beijing, China). cDNA (20 ng) was mixed with SYBR Green-MasterMix (Bio-Rad, Hercules, CA, USA) and amplified in CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. qRT-PCR was performed using a SYBR Premix EX Taq™ Kit (Takara, Dalian, China) by an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as the $2^{-\Delta\Delta Ct}$ values and are representative of at least three independent experiments. The primers used in the experiments were listed in Table I.

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. The absorbance of each well was measured at 450 nm wavelength. All experiments were performed in triplicate

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Transwell Invasion Assay

Cell migration and invasion assays were performed using Matrigel-coated (invasion assay 24-well transwell plates (8-μm pore size) (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, cells suspended in 200-μl serum-free 1640 medium were placed into the upper chamber of the insert with or without Matrigel. After 24 h of incubation, cells remaining on the upper membrane were carefully removed. Five visual fields of each insert were randomly chosen and photographed under a light microscope at ×200 magnification. All experiments were performed in triplicate.

Luciferase Assays

To confirm that miR-212 can bind to the predicted site TCF7L2, we used TargetScan online software and miRanda to confirm miR-212 targeting TCF7L2. Next, Reporter constructs containing pGL3- TCF7L2 and pGL3-mut TCF7L2 (with a mutated target seed sequence) were obtained from Bio-Asia (Wuhou, Chengdu, China). Cervical cancer cells were co-transfected with the luciferase reporters and a test substance or vehicle using Lipofectamine 2000. Forty-eight hours after transfection, firefly, and renilla luciferase assays were performed using the Dual-Luciferase Reporter System (Promega, Dongcheng, Beijing, China) according to manufacturer instructions.

Table I. The primer sequence for RT-PCR.

Primer	Primer sequence (5′-3′)
miR-212-F	CGCTAACAGTCTCCAGTC
miR-212-R	GTGCAGGGTCCGAGGT
GAPDH-F	AGCCACATCGCTCAGACAC
GAPDH-R	GCCCAATACGACCAAATCC

Western Blot

Western blotting was used to further determine the expression differences of TCF7L2 after transfection with miR-212 and anti-miR-212. The cell lysates were boiled with loading buffer at 95°C for 5 min, separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 80 V for 2 h and, then, transferred to PVDF membranes for 2 h. After blocking nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with a primary rabbit anti-human TCF7L2. After being blocked in 5% nonfat milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) for 2 h at room temperature, the membranes were incubated overnight at 4°C with the corresponding primary antibodies. The experiments were carried out on three separate occasions.

Statistical Analysis

Data were analyzed using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA). Differences between the two groups were compared using the independent-samples t-test, χ^2-test, or Fisher's exact test, as appropriate. p-value < 0.05 was considered as statistically significance.

Results

miR-212 was Down-regulated in Cervical Cancer Tissues and Cell Lines

We performed quantitative PCR analysis to detect the expression level of miR-212 in cervical cancer tissues and cell lines. Our results showed that miR-212 is down-regulated in cervical cancer tissues compared to paired adjacent normal tissues (Figure 1, $p < 0.01$). We next examined miR-212 expression in four human cervical cancer cell lines (HeLa, SiHa, CaSKi, and C33A) and End1/E6E7 cells by quantitative PCR. It was shown that the expression level of miR-212 was significantly lower in cervical cancer lines (HeLa, SiHa, CaSKi, and C33A) than in the adjacent normal tissues (Figure 2).

MiR-212 Inhibits the Proliferation and Invasion of HeLa and SiHa Cells

To investigate whether miR-212 is related to tumor proliferation and metastasis, we detected the proliferation and invasion capacity of cervical cancer cells by BrdU assays. HeLa and SiHa were transfected with miR-212 mimics. The increased levels of miR-212 can be detected in the HeLa and SiHa cells after transfection (Figure 2A-2B). Next, Compared with the negative control cells, the miR-212-transfected cells grew markedly slower (Figure 2C-2D). Last, we also found that overexpression of miR-212 decreased invasiveness of HeLa and SiHa cells (Figure 2E-2F). On the other hand, transfection with miR-212 inhibitor led to a significant raise in cell proliferation and invasion in both cells (Figure 3A-3F). These results indicated that miR-212 functions as a negative regulator of cervical cancer cell growth and metastasis.

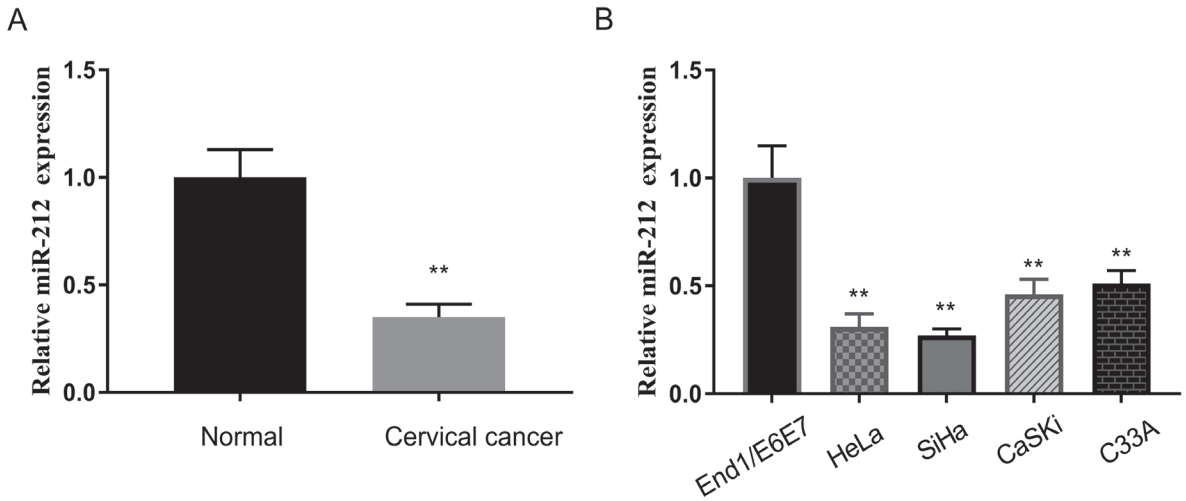

Figure 1. Expression of miR-212 in cervical cancer tissues and cell lines. ***A,*** miR-212 expression was significantly lower in cervical cancer tissues than in the corresponding non-cancerous tissues. ***B,*** miR-212 expression was downregulated in the cervical cancer cell lines HeLa, SiHa, CaSKi and C33A compared with normal cervical epithelial cell line (End1/E6E7). *$p < 0.05$, **$p < 0.01$.

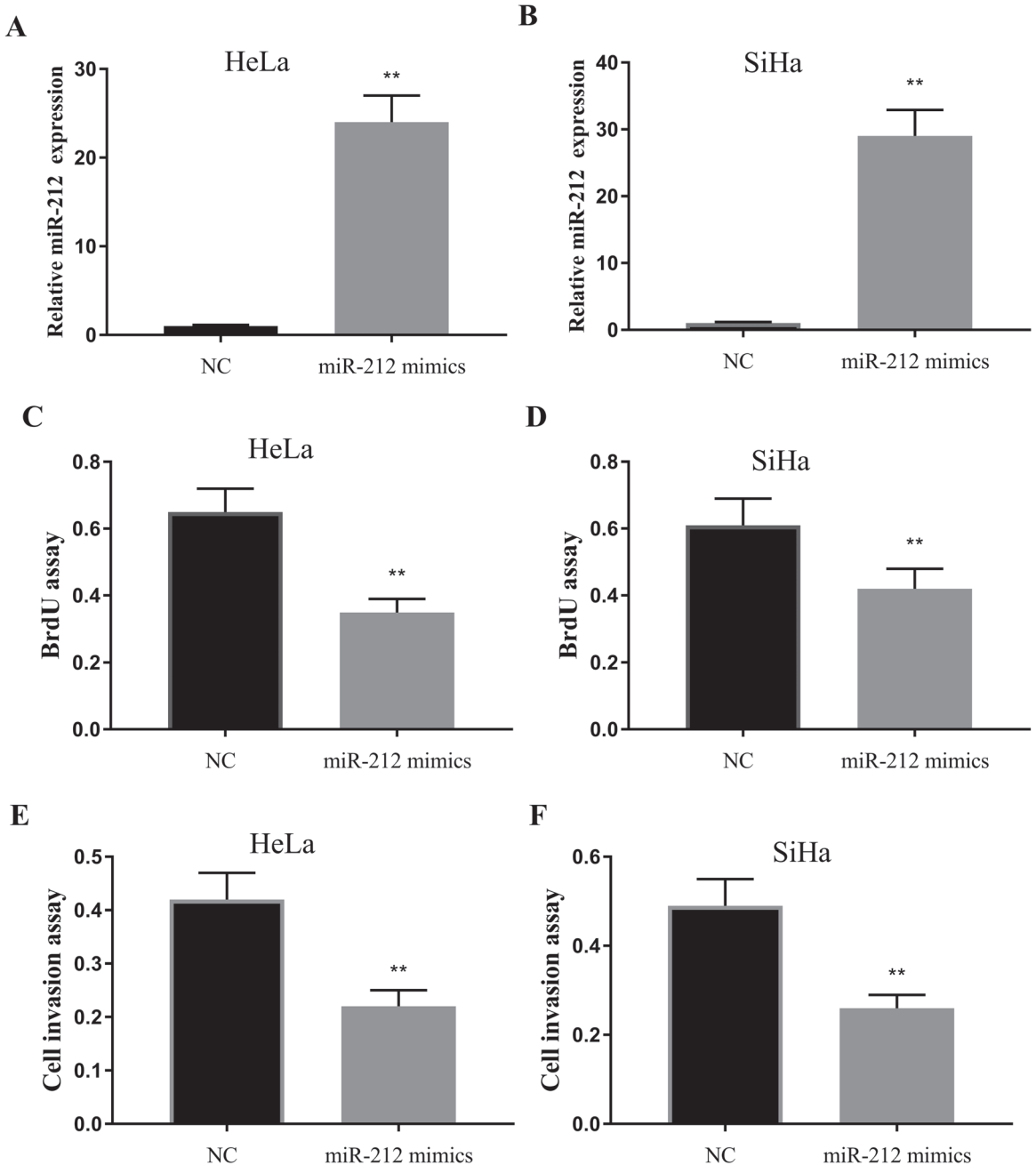

Figure 2. Ectopic expression of miR-212 inhibited cell proliferation and invasion. ***A-B,*** The relative expression levels of miR-212 in HeLa and SiHa cells were determined by RT-qPCR after transfection with miR-212 mimics or miR-NC. ***C-F,*** The proliferative cell potential (BrdU) and invasion abilities were determined in HeLa and SiHa cells transfected with miR-212 mimics or NC. *$p < 0.05$, **$p < 0.01$.

TCF7L2 is a Direct and Specific Target of miR-212

To investigate the mechanisms by which miR-212 influences cellular proliferation and invasion, we employed TargetScan s to search for putative protein coding gene targets of miR-212, and identified TCF7L2 as a possible target of miR-212 (Figure 4A). Luciferase reporter assays were adopted to investigate whether the 3'UTR of TCF7L2 could be directly targeted by miR-212. The result showed that, in Hela cells transfected with luc-TCF7L2-WT, there was a

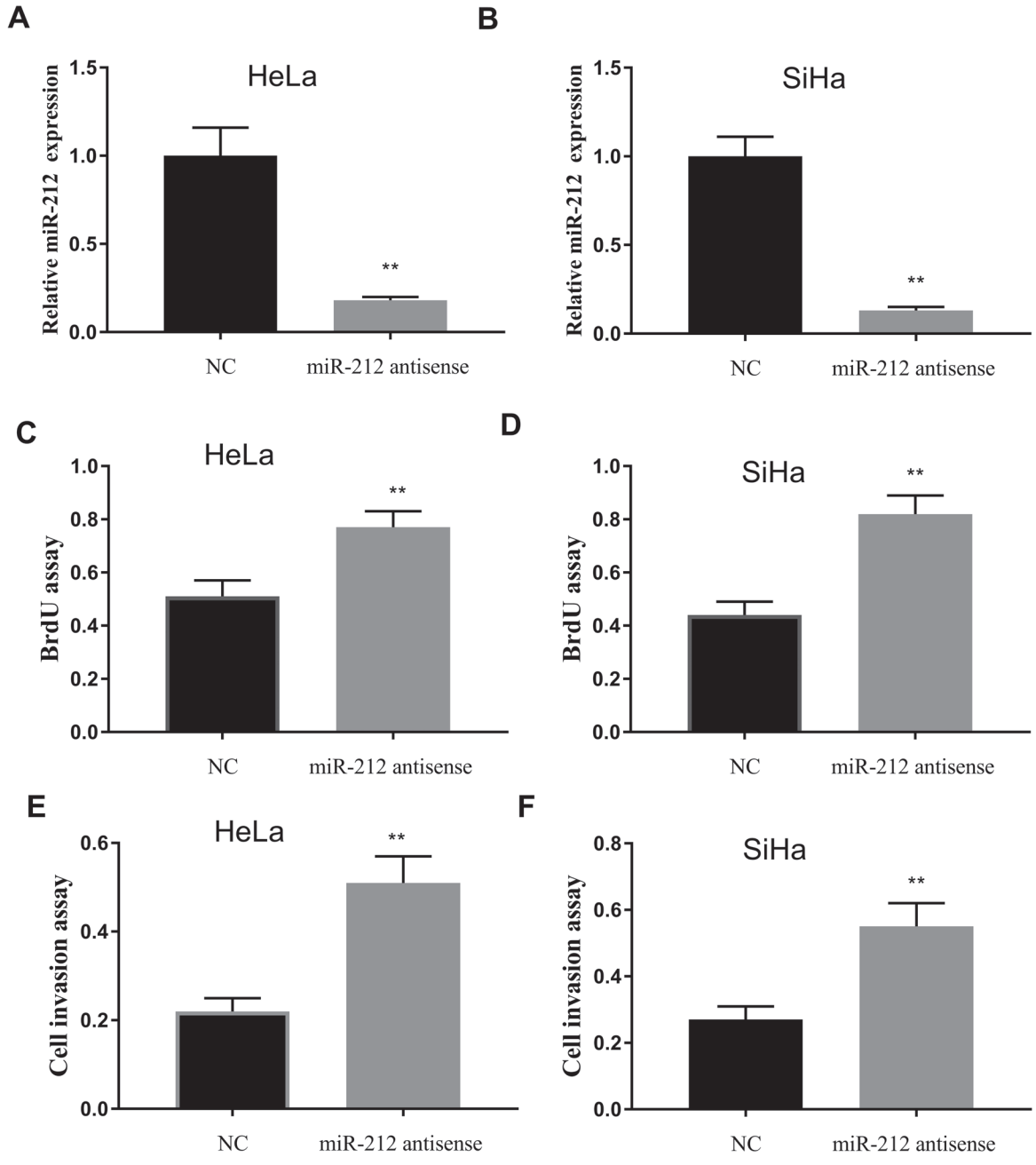

Figure 3. Downregulation of miR-212 promoted cervical cancer cell proliferation and invasion. ***A-B,*** The relative expression levels of miR-212 in HeLa and SiHa cells were determined by RT-qPCR after transfection with miR-212 antisense or miR-NC. ***C-F,*** The proliferative cell potential (BrdU) and invasion abilities were determined in HeLa and SiHa cells transfected with miR-212 antisense or NC. *$p < 0.05$, **$p < 0.01$.

significant difference in relative luciferase activity between miR-NC and miR-212-mimics co-transfection. However, in Hela cells transfected with luc-TCF7L2-MT, there was no difference in relative luciferase activity between miR-NC and miR-212-mimics co-transfection (Figure 4B). Next, Western blot results revealed that overexpression of miR-212 remarkably reduced the expression of TCF7L2 in both Hela and SiHa cells (Figure 4C-4D). In contrast, down-expression of miR-212 remarkably enhanced the expression of TCF7L2 in both Hela and SiHa

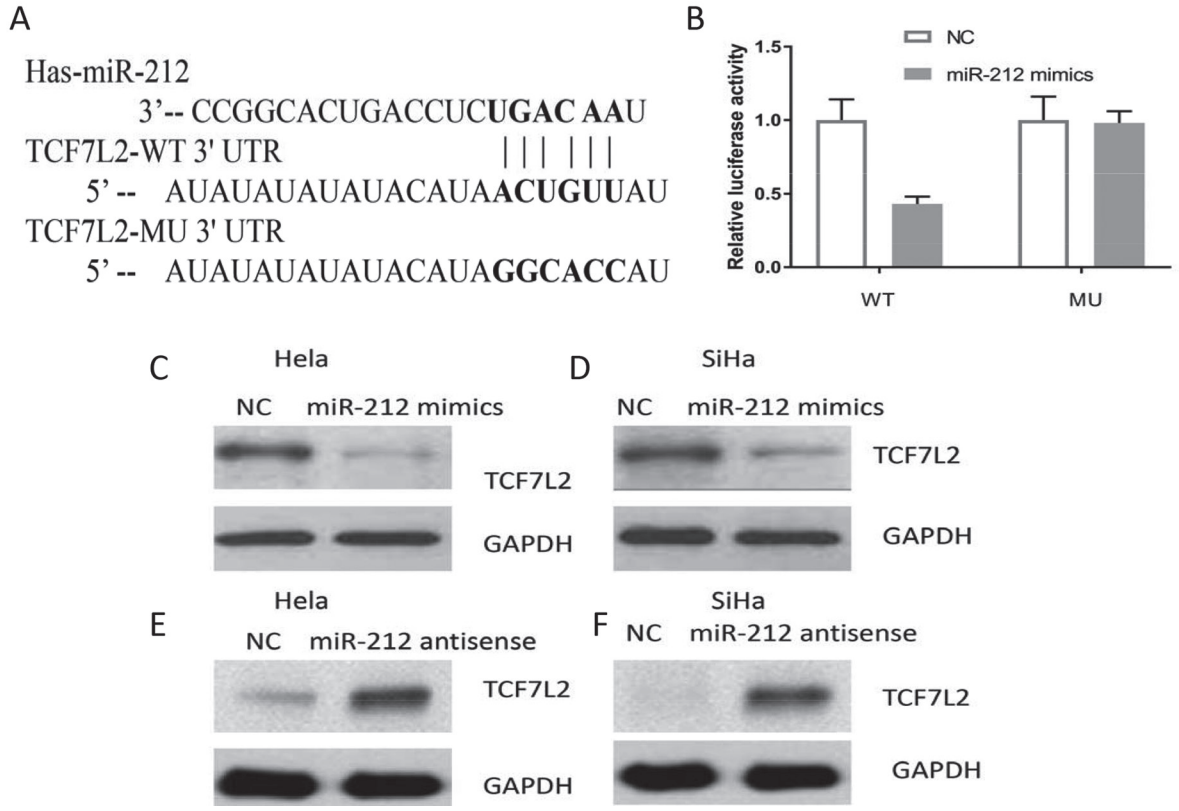

Figure 4. MiR-212 downregulates TCF7L2 expression through targeting its 3'UTR. ***A,*** TCF7L2 was predicted to be a target gene of miR-212 by TargetScan. ***B,*** Luciferase activity of wild-type (UTR-WT) or mutant (UTR-mut.) TCF7L2 3′-UTR reporter gene in HeLa cells infected with the miR-212. ***C-F,*** Protein expression levels of TCF7L2 in HeLa and SiHa with miR-212 andanti-miR-212 transfection. *$p < 0.05$, **$p < 0.01$.

cells (Figure 4E-4F). Taken together, our results strangely suggested that miR-212 played its role in suppressing cervical cancer growth via inhibiting TCF7L2 expression.

Discussion

Cervical cancer is caused by a series of genetic and epigenetic alterations in which miRNAs play a crucial role[13]. miRNAs involved in carcinogenesis and progression of cervical cancer have been widely investigated[14]. Invasion and metastasis are major reasons for the poor prognoses of cervical cancer patients. Although previous studies have indicated that many molecular mechanisms contribute to the metastasis of cervical cancer[15,16], the molecular mechanisms of cervical cancer and progression remain poorly understood. A better understanding of the molecular events underlying the cervical cancer metastasis is important for its prevention and treatment.

MiR-212, which is located at chromosome 17p13.3, has been shown to be deregulated in various human cancers. Previous researches revealed that miR-212 played a different role in different tumor types. For instance, miR-212 functioned as a tumor suppressor in hepatocellular carcinoma[17], gastric cancer[18], and osteosarcoma[19]. However, miR-212 served as a tumor promoter in esophageal cancer[20], colorectal cancer[21], and prostate cancer[22]. For cervical cancer, Zhao et al[12] found that miR-212 expression was are downregulated in human cervical cancer tissues and cell lines and overexpression of miR-212 suppressed proliferation and metastasis of cervical cancer by targeting SMAD2, suggesting that miR-212 served as a tumor suppressor in cervical cancer. Previous studies[23,24] have identified Wnt/β-catenin signaling as a direct and functional target of several miRNAs. TCF7l2 has been identified as a new transcriptional factor to promote EMT in tumour cells, and it is also the key factor in the Wnt signalling pathway[25,26]. Several reports[27,28] revealed that miRNAs

could regulate the expression of TCF7l2. Those results revealed that TCF7l2 played an important oncogene role in tumor.

In the present work, we analyzed expression of miR-212 in different cervical cancer cell lines and tissues and found that the expression levels of miR-212 were downregulated in both cervical tissues and cell lines. Our cell experiments showed that up-regulated miR-212 in cervical cancer cells could inhibit proliferation and invasion, while its down-regulation can promote proliferation and invasion in non-cervical cancer cells. To experimentally validate whether TCF7l2 was a target gene of miR-212 in cervical cancer, we performed dual luciferase assays. The result showed that TCF7l2 was the targeted gene of miR-212. Furthermore, meanwhile, Western blot analysis confirmed that overexpression of miR-212 downregulated the expression of TCF7l2.

Conclusions

The present study showed the tumor suppressor function of miR-212 was mediated by the downregulation of its downstream target gene TCF7l2. We consider that miR-212 may be a therapeutic target in cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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