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Long non-coding RNA WT1-AS inhibits cell aggressiveness via miR-203a-5p/FOXN2 axis and is associated with prognosis in cervical cancer

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Abstract. – OBJECTIVE: Substantial evidence has demonstrated that long non-coding RNAs (lncRNAs) play pivotal roles in tumorigenesis and tumor progression. The lncRNA Wilms tumor 1 Antisense RNA (WT1-AS) is a potential tumor suppressor in some types of cancers. The objective of this study was to evaluate the biological roles of WT1-AS in cervical cancer.

PATIENTS AND METHODS: The Cancer Genome Atlas (TCGA) was used to identify differentially expressed lncRNAs in cervical carcinoma. The level of lncRNA WT1-AS in cervical carcinoma tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The lentiviral vector encoding WT1-AS (LV-WT1-AS) or miR-203a-5p mimic was transfected into cervical carcinoma cells. Cell Counting Kit-8 (CCK-8), wound healing and transwell invasion assays were applied to assess the role of WT1-AS in cervical cancer cell growth and migration. WT1-AS directly bound to miR-203a-5p was confirmed using Luciferase reporter assay. The level of forkhead box N2 (FOXN2) was assessed by quantitative Real Time-Polymerase Chain Reaction analysis. A xenograft model was constructed to explore the role of WT1-AS in cervical cancer cell growth *in vivo*.

RESULTS: WT1-AS was down-regulated in both cervical cancer tissues and cell lines. Functional analyses indicated that the over-expression of WT1-AS remarkably inhibited cervical carcinoma cell growth, migration and invasion. The results of the Luciferase reporter assays verified that miR-203a-5p is a direct target of WT1-AS. Moreover, FOXN2 was identified as a direct target gene of miR-203a-5p, and the up-regulation of miR-203a-5p reversed the inhibitory effects of WT1-AS in cervical cancer cells.

CONCLUSIONS: Our results demonstrated that WT1-AS was under-expressed in cervical carcinoma and suppresses cervical cancer cell growth and aggressiveness via a miR-203a-5p/FOXN2 axis.

Key Words:
WT1-AS, MiR-203a-5p, FOXN2, Cervical cancer.

Introduction

Cervical carcinoma is a common malignant cancer among females and remains the leading cause of cancer-related deaths among women worldwide[1,2]. Although the diagnostic and therapeutic options for cervical cancer have markedly advanced in the past few years, the overall survival of advanced cervical cancer remains unfavorable due to metastasis[3]. Hence, studies of the biological mechanism of cervical carcinoma metastasis are urgently needed[4]. Long non-coding RNAs (lncRNAs) are important members of the non-coding RNA family[5]. Currently, lncRNA is defined as transcriptional "noise" because of its non-coding characteristic. A growing number of studies demonstrate that lncRNAs play pivotal roles in many biological processes[6]. Substantial evidence suggests that lncRNAs are aberrantly expressed in multiple cancers, including gastric carcinoma[7], colon cancer[8] and breast carcinoma[9], and are associated with overall survival. Wilms tumor 1 Antisense RNA (WT1-AS) is an antisense tran-

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script of the Wilm's tumor gene, which encodes a zinc finger transcription domain with either tumor suppressor or oncogenic activities[10]. WT1-AS has been found to be aberrantly expressed in several malignant tumors[11-13]. The expression of WT1-AS is remarkably down-regulated in gastric cancer samples compared to the corresponding normal tissues. The growth, migration and invasion of gastric cancer cells are inhibited after WT1-AS is ectopically expressed in gastric carcinoma cells[14]. Moreover, the WT1-AS promoter has been found to be significantly methylated in ovarian clear cell adenocarcinoma (OCCA), which suggests the potential role of WT1-AS in the prognosis of patients with OCCA[11]. However, the role of lncRNA WT1-AS in regulating cervical cancer invasion and metastasis has not yet been thoroughly investigated. In this work, we revealed that WT1-AS is under-expressed in cervical cancer, including clinical specimens and cell lines. With the over-expression of WT1-AS, cervical cancer cell growth, mobility and invasion were significantly inhibited, and cervical carcinoma cell growth was also suppressed. Furthermore, we demonstrated that the up-regulation of WT1-AS markedly decreased the level of miR-203a-5p in cervical carcinoma cells. Both bioinformatics and Luciferase reporter analysis suggested that miR-203a-5p binds to WT1-AS in a sequence-specific manner. Regarding the mechanism, miR-203a-5p directly targeted the mRNA of FOXN2 in cervical cancer cells. Finally, we found that WT1-AS inhibited cervical cancer cell progression and regulated FOXN2 expression via sponging miR-203a-5p. In summary, we showed that a WT1-AS/miR-203a-5p/FOXN2 axis regulates cell growth and metastasis in cervical cancer, which may serve as a potential target for cervical cancer treatment.

Materials and Methods

Clinical Specimens and Cervical Cancer Cell Lines

Forty-seven cases of cervical cancer samples and para-tumor tissue were obtained from The First Hospital of the Jilin University. After surgical resection, the specimens were stored immediately in liquid nitrogen until RNA extraction. This study was approved by the Ethics Committee of The First Hospital of the Jilin University. Informed consent from patients was obtained. Cervical carcinoma cells (SiHa, C-4-I, Ca-Ski and C-33-A), 293T and normal cervical epithelial cell (ECT1/E6E7) were purchased from the Nanjing Cobioer Biotech Co., Ltd. (Nanjing, Jiangsu, China). SiHa, C-4-I, C-33-A and ECT1/E6E7 cells were cultured in Eagle's Minimum Essential Medium (EMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Ca-Ski cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS; Gibco, Grand Island, NY, USA). 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA, USA). All cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

Cell Transfection

Lentiviral vector encoding WT1-AS (LV-WT1-AS) and its control (LV-Vector) were purchased from GeneCopoeia (Rockville, MD, USA). MiR-203a-5p mimic (miR-203a-5p) and miRNA mimic negative control (miR-ctr) were obtained from GeneCopoeia (Rockville, MD, USA). Lentiviral vector or miRNA transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from clinical tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The level of WT1-AS was measured using SYBR Premix EX Taq™ II kit (TaKaRa, Otsu, Shiga, Japan) on ABI Prism®7500 (Applied Biosystems, Foster City, CA, USA). MiRNA was collected using PureLink™ miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA), and its level was measured using TaqMan microRNA assay kit. The relative level of miRNA and lncRNA were calculated using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal controls. The sequences of the primers were the following: WT1-AS-F: GCCTCTCTGTCCTCTTCTTTGT; WT1-AS-R: GCTGTGAGTCCTGGTGCTTAG; GAPDH-F: GCTCTCTGCTCCTCCTGTTC; GAPDH-R: ACGACCAAATCCGTTGACTC; miR-203a-5p-F: GUGAAAUGUUUAGGACCACUAG; miR-203a-5p-R: AGUGGUCCUAAACAUUUCACUU; U6-F: CTCGCTTCGGCAGCACA; and U6-R: AACGCTTCACGAATTTGCGT.

Cell Proliferation Assay

Briefly, SiHa or Ca-Ski cells were placed into 96-well plates after transfection with LV-WT1-AS or LV-WT1-AS plus miR-203a-5p. Cells were cultured for 24, 48, 72 and 96 h. Then, Cell Counting Kit-8 reagents (CCK-8; Beyotime, Shanghai, China) were added to the 96-well plate, and it was incubated for 2 h. The absorbance at 450 nm was detected using a microplate reader.

Cell Migration Assay

Cells were seeded into a 6-well plate and cultured for 24 h. The cell monolayer was scratched using a 100 µl tip, and cells were then cultured with serum-free medium. The images of a gap at 0 h and 24 h were collected using a standard microscope, and the percentage of wound closure was calculated[15].

Invasion Assay

Cells were seeded into transwell inserts (Corning, Lowell, MA, USA) with a polycarbonate membrane (8 µm) pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After 24 h, the cells that invaded the lower chamber were fixed and stained with 1% crystal violet. The invading cells in five fields of view were counted[16].

Luciferase Reporter Assay

The wide-type (wt) fragment of WT1-AS containing miR-203a-5p binding sites or the mutant fragment (mut) was synthesized by GeneCopoeia (Guangzhou, Guangdong, China) and inserted into the pmirGLO Luciferase reporter vector (Promega, Madison, WI, USA). The wild-type (wt) 3'-UTR of FOXN2 with putative miR-203a-5p binding sites or the mutant sequence (mut) was synthesized by GeneCopoeia (Rockville, MD, USA) and cloned into the pmirGLO vector. 293T cells were co-transfected with the pmirGLO vector and miR-203a-5p or miR-ctr. The Luciferase reporter assay system (Promega, Madison, WI, USA) was used to detect Luciferase activity.

In Situ Hybridization

WT1-AS expression in cervical cancer tissue was analyzed using a biotin-labeled specific WT1-AS probe. Paraffin sections were deparaffinized with xylene and 100% ethanol. Then, the sections were incubated with biotin-labeled probes for 18 h at 40°C. DAB substrate was used for colorimetric detection of WT1-AS. Finally, the sections were co-stained with hematoxylin, followed by dehydration in graded alcohols and xylene.

Immunofluorescence Staining

Cells grown on glass coverslips were fixed using pre-chilled acetone, and then incubated with 1% of Bovine Serum Albumin/Phosphate-Buffered Saline (BSA/PBS) to avoid non-specific binding. After that, cells were incubated with the anti-FOXN2 antibody (1:500, Epitomics, Burlingame, CA, USA) overnight at 4°C. After washing with pre-chilled PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:100, Boster Biotechnology, Wuhan, Hubei, China). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China). Images were taken using a Zeiss inverted microscope (Carl Zeiss, Hallbergmoos, Germany).

Xenograft Model of Cervical Cancer Cell Growth

The mouse experiments were conducted following the standard operating procedures approved by the Committee on the Use and Care of Animals from The First Hospital of the Jilin University. LV-WT1-AS or empty vector transfected SiHa cells were subcutaneously inoculated into nude mice. The tumor size was measured each week using a caliper, and the tumor volume was calculated as follows: length x width^2/2.

Statistical Analysis

All data are shown as the mean ± standard deviation (SD). GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis. The differences between the two groups were evaluated using either two-tailed Student's *t*-test or one-way ANOVA followed by Post-Hoc Dunnett's test. $p < 0.05$ was considered as statistical significance.

Results

LncRNA WT1-AS is Under-Expressed in Cervical Cancer

Gene expression datasets of cervical cancer used for statistical analysis were acquired from The Cancer Genome Atlas (TCGA). The screening was performed in both cervical cancer and matched normal tissues. As shown in Figure 1A-1B, WT1-AS was one of the most under-expressed lncRNAs in cervical carcinoma tissues. Then, we performed *in situ* hybridization assays to further measure the expression of WT1-AS in cervical cancer tissues. We found that the level of WT1-AS

Table I. The association between WT1-AS expression and clinic-pathological factors in cervical cancer patients.

Clinical parameter	WT1-AS High	Low	*p*-value
Age (years)			0.068
≤40	17	10	
>40	11	9	
Size (cm)			
≥4	14	11	0.077
<4	12	10	
FIGO stage			
I	8	13	0.009
II-III	9	17	
Lymphatic metastasis			
Yes	5	13	0.005
No	19	10	

FIGO: International Federation of Gynecology and Obstetrics.

was lower in tumor tissue than in paired peri-tumor tissue (Figure 1C). To further explore the expression pattern of lncRNA WT1-AS in cervical cancer, quantitative Real Time-Polymerase Chain Reaction (RT-PCR) analyses were conducted in 47 cervical carcinoma samples and corresponding non-tumor tissues. As shown in Figure 1D, WT1-AS was markedly decreased in cervical carcinoma samples compared to that in the matched non-tumor tissues. Moreover, we compared the level of WT1-AS between patients with different clinical stages of cervical cancer and found that the level of WT1-AS was relatively lower in stage II-III than in stage I (Figure 1E). Meanwhile, WT1-AS was markedly lower in cervical carcinoma patients with lymph node metastasis than those without metastasis (Figure 1F). Finally, the Kaplan-Meier analysis of 47 patients with cervical cancer suggested that patients who had a lower level of WT1-AS exhibited a poor overall survival (Figure 1G and Table I). Consistently, the expression profiles of WT1-AS were detected in four cervical cancer cell lines by quantitative Real Time-Polymerase Chain Reaction. The results showed that WT1-AS was down-regulated in all cervical cancer cell lines (SiHa, C-4-I, Ca-Ski and C-33-A) compared to normal cervical epithelial cell line, ECT1/E6E7 (Figure 1H). These findings indicated that WT1-AS has low expression in cervical cancer and is closely associated with its progression.

Over-Expression of WT1-AS Decreases the Growth and Invasion of Cervical Cancer Cells

To explore the roles of WT1-AS in cervical cancer, we performed a series of functional analysis in SiHa and Ca-Ski cells. LV-WT1-AS plasmids were transfected into SiHa or Ca-Ski cells to overexpress WT1-AS. Additionally, LV-Vector plasmids were transfected as controls. The transfection efficiency was verified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and increased expression of WT1-AS was observed in SiHa and Ca-Ski cells (Figure 2A). Cell Counting Kit-8 (CCK-8) assays were applied to detect the cell proliferative ability. As shown in Figure 2B-2C, THE over-expression of WT1-AS inhibited the proliferation of Ca-Ski and SiHa cells. The wound closure and transwell invasion analyses revealed that WT1-AS over-expression significantly inhibited the migration and invasive ability of cervical carcinoma cells (Figure 2D-2E). These data suggested that the up-regulation of WT1-AS inhibited the growth, mobility and invasion of cervical cancer cells.

LncRNA WT1-AS Directly Binds to MiR-203a-5p

Due to its vital role in RNA homeostasis, an essential function of lncRNAs is to compete with endogenous RNA (ceRNA)[17]. To further identify the underlying mechanism by which WT1-AS regulates cervical cancer progression, the online bioinformatics tool RNA22 (http://34.236.212.39/microrna/getGeneForm.do) was used to predict the targets of WT1-AS. As shown in Figure 3A, miR-203a-5p was a predicted target of WT1-AS with potential binding sites. The expression of miR-203a-5p in cervical cancer cells transfected with LV-WT1-AS was explored. The results showed that the level of miR-203a-5p was remarkably reduced in cells overexpressing WT1-AS (Figure 3B). Next, SiHa and Ca-Ski cells were transfected with miR-203a-5p mimics, which significantly suppressed the expression of WT1-AS (Figure 3C-3D). To confirm that WT1-AS binds to miR-203a-5p directly, Luciferase reporter assays were executed. The fragment of WT1-AS containing putative miR-203a-5p binding sites (wt-WT1-AS) or mutant fragment (mt-WT1-AS) was inserted into the pmirGLO Luciferase reporter vector. Then, the Luciferase reporter vector combination with miR-203a-5p or miR-ctr was co-transfected into 293T cells. Luciferase activity was decreased in 293T cells transfected with miR-203a-5p and wt-WT1-AS, whereas the suppressive effects of miR-203a-5p were abolished in cells transfected with mt-WT1-AS (Fig-

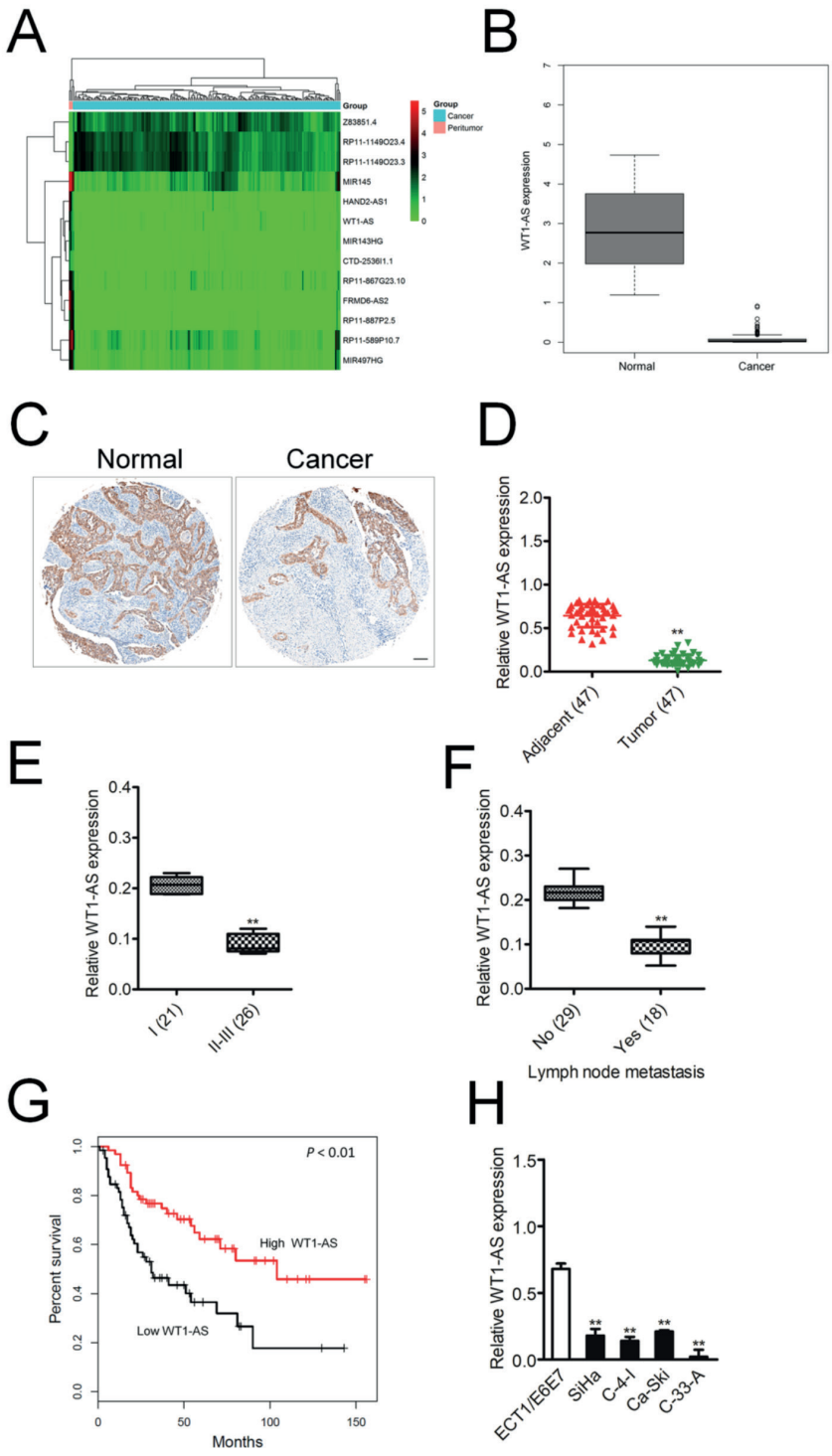

Figure 1. WT1-AS inhibits cervical cancer cells growth. ***A,*** The representative down-expressed lncRNAs in cervical cancer as compared to non-cancer tissues was summarized in the heat map. ***B,*** The level of WT1-AS was remarkably low-expressed in cervical cancer compared to normal. ***C,*** The expression of WT1-AS in cervical cancer tissues was checked by *in situ* hybridization. Scale bar: 100 μm. ***D,*** The level of WT1-AS in the para-tumor and tumor tissues from patients with cervical cancer was determined by qRT-PCR assay. $^{**}p < 0.01$, compared to adjacent. ***E,*** The levels of WT1-AS in metastatic and non-metastatic cervical cancer tissues were shown. $^{**}p < 0.01$, compared to no metastasis. ***F,*** The levels of WT1-AS in I or II-III tumor stage were shown. $^{**}p < 0.01$, compared to I. ***G,*** Overall survival analysis of cervical cancer patients with high or low level of WT1-AS. ***H,*** The level of WT1-AS in cervical cancer cell lines and ECT1/E6E7 cells was detected by qRT-PCR. $^{**}p < 0.01$, compared to ECT1/E6E7 cells.

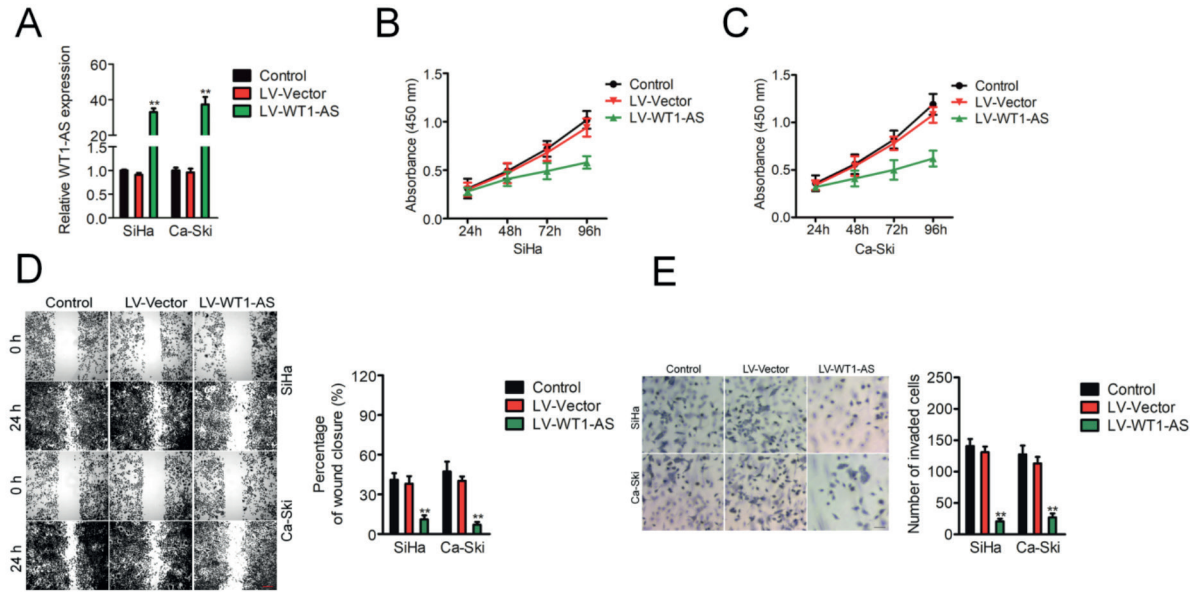

Figure 2. WT1-AS inhibits cervical cancer cells growth, migration and invasion. ***A,*** Ca-Ski and SiHa cells were transfected with LV-WT1-AS or LV-Vector, and the level of LV-WT1-AS was detected by qRT-PCR. ***B-C,*** WT1-AS suppressed the growth of SiHa and Ca-Ski. ***D,*** WT1-AS inhibited the migratory activities of both SiHa and Ca-Ski cells. ***E,*** WT1-AS repressed the invasive activities of both SiHa and Ca-Ski. $^{**}p < 0.01$, compared to control.

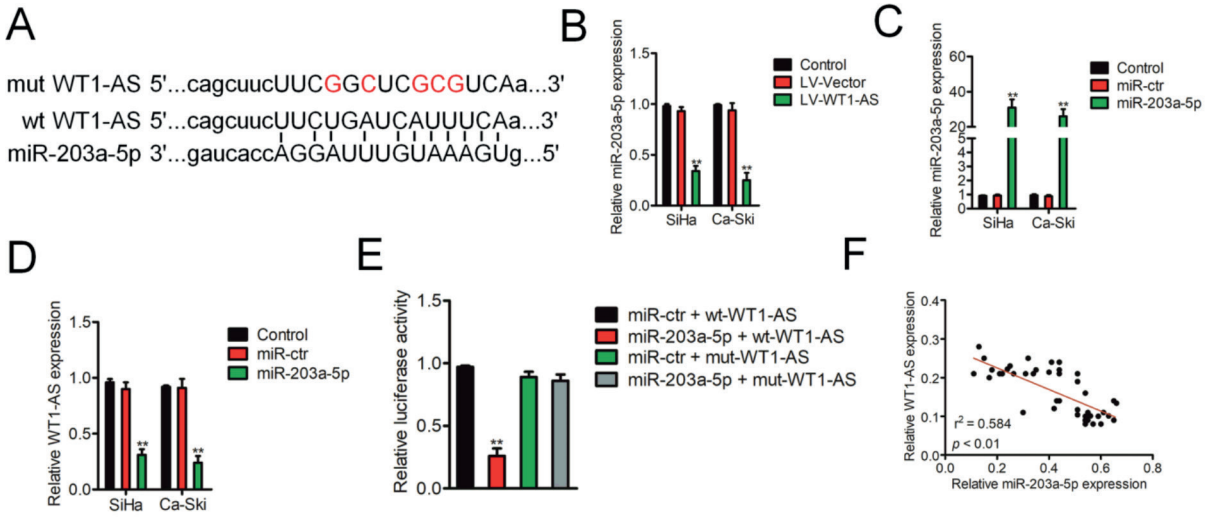

Figure 3. LncRNA WT1-AS directly binds to miR-203a-5p. ***A,*** The potential binding sites between WT1-AS and miR-203a-5p was identified using RNA22. ***B,*** WT1-AS inhibited the level of miR-203a-5p in SiHa and Ca-Ski cells. ***C,*** Cells were transfected with miR-203a-5p or miR-ctr, and the level of miR-203a-5p was detected by qRT-PCR assay. ***D,*** MiR-203a-5p inhibited the expression of WT1-AS in SiHa and Ca-Ski cells. $^{**}p < 0.01$, compared to control. ***E,*** Luciferase reporter assay demonstrated that WT1-AS directly targeted miR-203a-5p. ***F,*** Association between WT1-AS and miR-203a-5p in cervical cancer tissues was evaluated. $^{**}p < 0.01$, compared to miR-ctr + wt-WT1-AS.

ure 3E). The detection of miR-203a-5p in cervical carcinoma tissues by qRT-PCR suggested that miR-203a-5p is negatively associated with WT1-AS (Figure 3F). Therefore, lncRNA WT1-AS directly binds to miR-203a-5p.

WT1-AS Exerts its Function Via MiR-203a-5p/FOXN2

The online bioinformatics tool Target Scan (http://www.targetscan.org/vert_71/), which was used to identify targets of miR-203a-5p, predict-

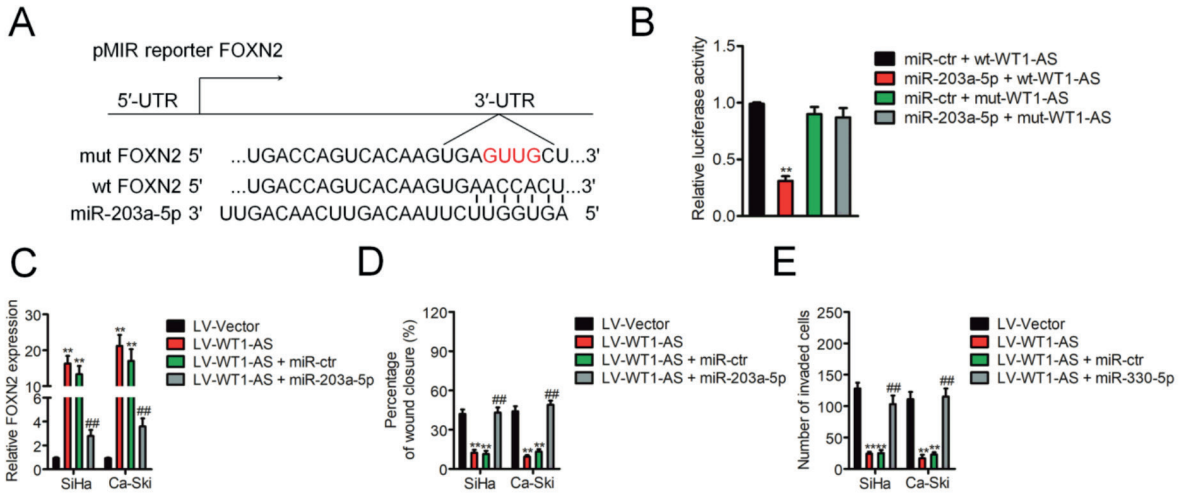

Figure 4. LncRNA WT1-AS exerts its inhibitory functions via miR-203a-5p/FOXN2 axis. ***A,*** A potential binding site was identified between FOXN2 and miR-203a-5p. ***B,*** Luciferase reporter assay demonstrated that miR-203a-5p directly targeted FOXN2. ***C,*** SiHa and Ca-Ski cells were co-transfected with LV-WT1-AS and miR-203a-5p or miR-ctr. The mRNA level of FOXN2 was determined by qRT-PCR. ***D,*** Repressive effects of WT1-AS on cervical cancers cells migration was attenuated by miR-203a-5p. ***E,*** The repressive effects of WT1-AS on cervical cancers cells invasion was attenuated by miR-203a-5p. $^{**}p < 0.01$, compared to LV-Vector; $^{\#\#}p < 0.01$, compared to LV-WT1-AS + miR-203a-5p.

ed that FOXN2 interacts with miR-203a-5p at their potential binding sequences (Figure 4A). A Luciferase reporter assay was performed to prove the interaction between FOXN2 and miR-203a-5p. Luciferase activity was decreased in 293T cells co-transfected with miR-203a-5p and the wild-type 3'-UTR of FOXN2 (wt-FOXN2). Nevertheless, the Luciferase activity was not inhibited in cells transfected with the mutant 3'-UTR of FOXN2 and miR-203a-5p (Figure 4B). To confirm if the effects of WT1-AS on cervical carcinoma cells were mediated by miR-203a-5p/FOXN2, SiHa and Ca-Ski cells were co-transfected with LV-WT1-AS and miR-203a-5p or miR-ctr. The qRT-PCR assay suggested that the expression of FOXN2 was enhanced by LV-WT1-AS but decreased by miR-203a-5p (Figure 4C). Furthermore, functional assays showed that the over-expression of miR-203a-5p diminished the inhibitory effects on cervical cancer cells induced by over-expressing WT1-AS (Figure 4D-E). These findings demonstrated that WT1-AS exerts its suppressive functions on cervical cancer cells through miR-203a-5p/FOXN2.

WT1-AS Suppresses Cervical Cancers Cell Growth in a Xenograft Model

To investigate the inhibitory effect of WT1-AS on the growth of cervical cancer cells *in vivo*, LV-WT1-AS or LV-Vector transfected SiHa cells were subcutaneously inoculated into nude mice to create a xenograft model. After four weeks of cell inoculation, WT1-AS markedly suppressed the growth of SiHa cells *in vivo* compared to LV-Vector transfected cells (Figure 5A). Moreover, transfection with WT1-AS remarkably reduced tumor weight (Figure 5B). Finally, tumor mass was analyzed by immunohistochemistry (IHC) staining with a Ki-67 antibody. As shown in Figure 4C, the level of Ki-67 was low in the tumor mass formed by LV-WT1-AS transfected cells in comparison with the LV-Vector transfected cells. Meanwhile, the level of miR-203-5p in the tumor mass formed by LV-WT1-AS transfected SiHa cells was significantly increased compared to the LV-Vector. Collectively, WT1-AS inhibited cervical cancer cell growth *in vivo*.

Discussion

Emerging evidence has demonstrated that lncRNAs play crucial functions in the regulation of physiology and pathology[18]. Previous studies prove that alteration of lncRNA expression affects cancer cells proliferation, apoptosis, invasion and the epithelial-mesenchymal transition (EMT) process[19]. For example, lncRNA H19 inhibits cells growth, mobility and invasion through the down-regulation of insulin receptor substrate

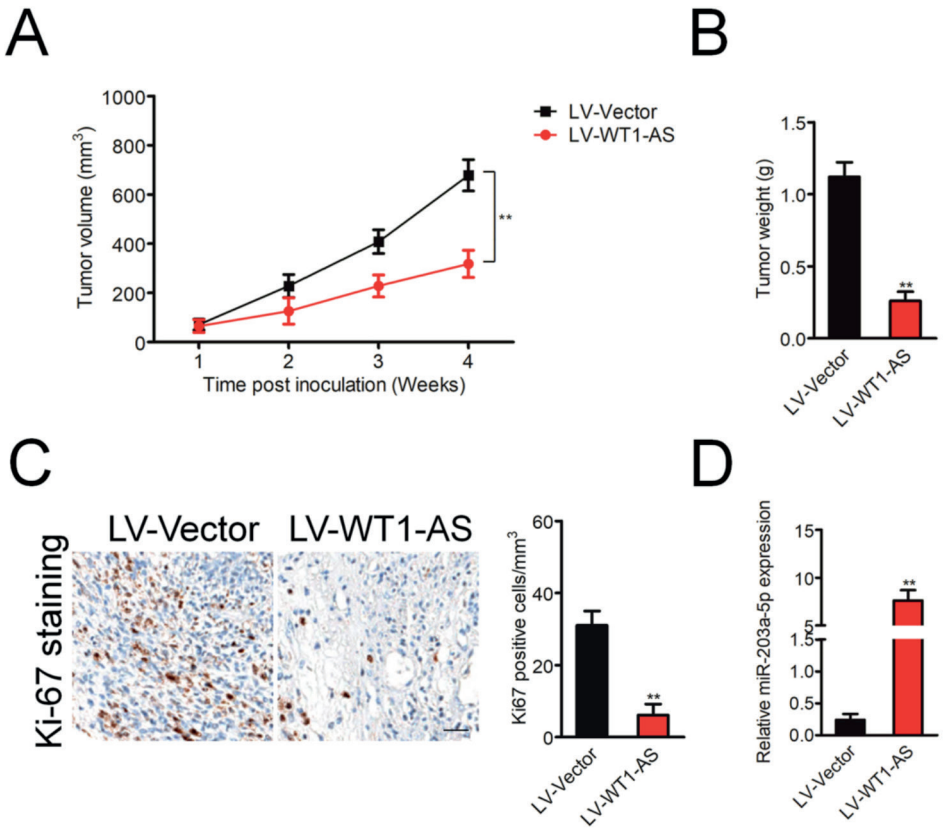

Figure 5. WT1-AS inhibits SiHa cells growth *in vivo*. ***A,*** Tumor growth was measured every week after inoculated with SiHa cells and tumor growth curve. ***B,*** After four weeks, the tumor was weighed. ***C,*** Representative immunohistochemical staining of Ki-67 in the SiHa xenografts. ***D,*** The level of miR-203q-5p in tumor mass was determined by qRT-PCR assay. $^{**}p < 0.01$, compared to LV-Vector.

1 (IRS-1) in thyroid cancer cells[20]. Long non-coding RNA (lncRNA)-activated by transforming growth factor-β (lncRNA-ATB) is up-regulated in osteosarcoma and positively associated with Enneking stage, metastasis and recurrence[21]. Meanwhile, the over-expression of lncRNA ATB promotes osteosarcoma cell growth and aggressiveness by inhibiting miR-200s[21]. In addition, lncRNA glutathione peroxidase 4 (GPX4) contributes to metastasis and regulates the EMT process in colorectal carcinoma through competing for miR-200b-3p, which modulates the expression of zinc finger E-box-binding homeobox 1 (ZEB1)[22]. Additionally, WT1-AS expression is significantly down-regulated in gastric carcinoma. The expression levels of WT1-AS are associated with tumor size and the clinicopathological stage of gastric carcinoma[14]. However, the underlying mechanism of WT1-AS in cervical carcinoma remains not well explored. Here, we report that WT1-AS is a metastasis-associated lncRNA that plays vital roles in regulating cervical carcinoma growth, migration and invasion. We found that WT1-AS was markedly down-regulated in cervical cancer and is associated with its progression. In addition, the over-expression of WT1-AS inhibited the aggressiveness of cervical cancer cells *in vitro*. Consistently, the over-expression of WT1-AS suppressed the growth of cervical cancer cells *in vivo*. In conclusion, our findings suggest that WT1-AS is a potential diagnostic biomarker and is a valuable therapeutic target for cervical cancer.

To explore the precise roles of WT1-AS in cervical cancer cells, Ca-Ski and SiHa cells were transfected with LV-WT1-AS to over-express WT1-AS. The over-expression of WT1-AS inhibited the growth of cervical cancer cells as demonstrated by the CCK-8 assay. After showing that WT1-AS suppressed cervical cancer cell proliferation *in vitro*, we investigated the role of WT1-AS on cervical cancer cell growth *in vivo*. Consistently, the up-regulation of WT1-AS remarkably decreased cervical cancer cell growth in the xenograft model. Metastasis is the primary cause of

death in patients with cancer. The initial steps of the metastatic cascade include local invasion, migration, EMT and intravasation[23]. LncRNAs are emerging as key regulators governing biological processes of metastasis, including migration and invasion. In this work, wound healing and transwell invasion assays were performed to explore whether WT1-AS regulates migration and invasion ability of cervical cancer cells. All the results suggested that migration and invasion ability of cervical cancer cells were remarkably inhibited by WT1-AS over-expression.

One of the functions of lncRNAs is to serve as competitive endogenous RNA (ceRNA) to sponge miRNAs and further regulate gene expression[24]. To explore whether WT1-AS acts as a miRNA sponge, a bioinformatics method was utilized, and the results suggested that there are binding sites for miR-203a-5p on WT1-AS. The Luciferase reporter and qRT-PCR activity assays confirmed that WT1-AS was negatively regulated by miR-203a-5p. It is well known that miRNAs regulate gene expression by binding to the 3'-UTR of the target gene[25,26,27]. In this work, bioinformatics was used to predict target genes, and FOXN2 was selected as the candidate target. The Luciferase reporter assay is a direct method for target validation that confirmed FOXN2 is a direct target gene of miR-203a-5p. Subsequently, miR-203a-5p reversed the role of WT1-AS on the growth and malignant phenotype of cervical cancer cells. The rescue experiments demonstrated that transfected miR-203a-5p markedly increased the cervical cancer cell growth and mobility that was inhibited by WT1-AS over-expression.

Conclusions

We showed that the level of lncRNA WT1-AS was down-regulated in cervical cancer specimens and cell lines. The over-expression of WT1-AS inhibited cervical cancer cell growth and aggressiveness via a miR-203a-5p/FOXN2 axis. Manipulation of miR-203a-5p reversed the functions of WT1-AS on the viability and motility of cervical cancer cells. Our study is the first to reveal the possible relationship between WT1-AS and miR-203a-5p in cervical cancer growth and metastasis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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