LncRNA HOXA-AS2 Facilitates Cervical Cancer Progression and Angiogenesis via miR-509-3p/BTN3A1 Axis

Ruxiang Chen
Guangzhou Women and Children's Medical Center

Ping He (✉ Heping-20088@hotmail.com)
Guangzhou Women and Children's Medical Center  https://orcid.org/0000-0002-2690-517X

Research article

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Abstract

**Background:** Cervical cancer, the leading cause of cancer-relevant mortality in females, is an aggressive malignant tumor. Tumor angiogenesis is vital for cell proliferation and metastasis in cancers. Accumulating studies have claimed that long non-coding RNAs (lncRNAs) participate in the progression of various cancers. The aim of this research is to explore the biological role and regulatory mechanism of LncRNA HOXA-AS2 in cervical cancer.

**Methods:** Experiments including RT-qPCR, western blot, RIP, MTT, EdU, transwell, luciferase reporter, RIP, FISH, tube formation assays were applied to investigate the biological role and regulatory mechanism of LncRNA HOXA-AS2 in cervical cancer.

**Results:** In current study, the results disclosed that HOXA-AS2 was notably upregulated in cervical cancer tissues and cell lines, and high HOXA-AS2 expression was strongly associated with poor prognosis of cervical cancer patients. Furthermore, HOXA-AS2 contributed to cell proliferation, migration, invasion and angiogenesis in cervical cancer. In addition, HOXA-AS2 absorbed miR-509-3p and miR-509-3p targeted BTN3A1 in cervical cancer. Besides, BTN3A1 overexpression partly rescued the inhibitory influence of HOXA-AS2 knockdown on cervical cancer progression and angiogenesis. Overall, HOXA-AS2 promoted cervical cancer progression and angiogenesis through sponging miR-509-3p to elevate BTN3A1 expression.

**Conclusions:** In other words, this paper was the first to study the molecular regulatory mechanism of HOXA-AS2 in cervical cancer and certified that HOXA-AS2 accelerated progression and angiogenesis in cervical cancer by targeting miR-509-3p/BTN3A1 axis, which may become a beneficial therapeutic target for cervical cancer.

Introduction

Cervical cancer is one of the most common cancers and the primary causes of cancer-related deaths in women worldwide with high incidence and mortality [1, 2]. Almost all cases of cervical cancer are caused by HPV infection. The main treatment options for cervical cancer patients include surgery and concurrent chemoradiotherapy. Cervical cancer kills more than a quarter of one million people a year in many developing countries because of inadequate treatment [3, 4]. Accordingly, it is of great necessity to explore the molecular mechanism of cervical cancer to improve its therapy.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs with over 200 nucleotides in length. They don't possess protein-coding potential [5, 6]. Extensive attention has been paid on lncRNAs for their significant roles in processes of multiple cancers, including cervical cancer. For example, lncRNA RBM5-AS1 exerts its oncogenic effect in oral squamous cell carcinoma by regulating miR-1285-3p/YAP1 axis [7]. LncRNA HLA-F-AS1 promotes the development of colorectal cancer through miR-330-3p/PFN1 axis [8]. LncRNA PCGEM1 boosts cell proliferation, migration and invasion via targeting miR-182/FBXW11 axis in cervical cancer [9]. LncRNA CRNDE enhances the tumorigenesis of cervical cancer through
absorbing miR-183 to modulating CCNB1 expression [10]. Moreover, it has been proved that IncRNA HOXA cluster antisense RNA 2 (HOXA-AS2) plays a carcinogenic role in plenty of tumors. For instance, HOXA-AS2 elevates the expression of SCN3A by binding with miR-106a to promote breast cancer development [11]. HOXA-AS2 contributes to the migration, invasion and stemness of bladder cancer cells by modulating miR-125b/Smad2 axis [12]. HOXA-AS2 facilitates initiation and progression of papillary thyroid cancer via targeting miR-15a-5p/HOXA3 axis [13]. Nevertheless, the biological role and molecular regulatory mechanism of HOXA-AS2 in cervical cancer remain to be clarified.

The aim of this paper was to probe the biological function and regulatory mechanism of HOXA-AS2 in cervical cancer. The results elucidated that HOXA-AS2 facilitates cervical cancer progression and angiogenesis via miR-509-3p/BTN3A1 axis, which may offer a novel perspective for cervical cancer diagnosis and therapy.

**Materials And Methods**

**Tissue samples**

Forty-six paired cervical cancer tissues and adjacent normal tissues were collected from the patients with cervical cancer at Guangzhou Women and Children's Medical Center. All these tissues were frozen in liquid nitrogen instantly and stored at -80°C until needed. Informed consent of this study was obtained from all patients and none of them received corresponding therapy of cervical cancer before operation. The study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center.

**Cell lines**

Four cervical cancer cell lines (HeLa, SiHa, C33A and CaSki) and one normal human cervical epithelial cell line Ect1/E6E7 were obtained from American Type Culture Collection (ATCC; USA) and cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humid incubator with 5% CO₂ at 37°C.

**Cell transfection**

Sh-HOXA-AS2#1, sh-HOXA-AS2#2, sh-NC, pcDNA3.1/BTN3A1, pcDNA3.1, miR-509-3p mimics, NC mimics were purchased from GenePharma (Shanghai, China). Cell transfections were accomplished with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

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

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen) following the manufacturer's recommendations. The reverse transcription of the extracted RNA into complementary DNA (cDNA) was performed with a Reverse Transcription Kit (Takara, Dalian, China). Then qRT-PCR was performed by utilizing SYBR Premix Ex Taq II Kit (Takara). Relative expressions of HOXA-AS2, miR-509-3p, and BTN3A1 were analyzed through the 2^−ΔΔCt method relative to GAPDH or U6 expression.
**Western blot assay**

Total proteins were isolated by using RIPA lysis buffer (Beyotime, Shanghai, China) with protease inhibitors (Roche, Shanghai, China) and the protein concentration was detected by a BCA Protein Assay Kit (Beyotime). Then proteins were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF; Millipore, USA) membranes. After being blocked in 5% skim milk for 2 hours at room temperature and cultured with primary antibodies overnight at 4°C, the membranes were washed by TBST solution and incubated with secondary antibodies for additional 2 hours at room temperature, followed by visualization with an ECL Detection System (Thermo Fisher Scientific, USA). The primary antibodies were as follows: VEGFA (ab32152, Abcam, Shanghai, China); Ang1 (ab8451, Abcam); FGF2 (ab92337, Abcam); BTN3A1 (ab236289, Abcam); GAPDH (ab8245, Abcam). GAPDH served as internal reference.

**3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay**

The treated cells were transferred into 96-well plates and cultured at 37°C. After 0, 24, 48 and 72 hours, 10 μl MTT solution was added to each well. Four hours later, 150 μl dimethyl sulphoxide (DMSO) was supplemented to every well. Then a microplate reader (BioRad, USA) was utilized to measure optical density (OD) value at 490 nm.

**5-ethynyl-2’-deoxyuridine (EdU) Assay**

To evaluate cell proliferation, EdU Detection Kit (Ribobio, Guangzhou, China) was utilized with the manufacturer’s protocols. HeLa or SiHa cells were seeded in 96-well plates. After incubation with 50 μM EdU labeling medium for 2 hours and fixation with 4% paraformaldehyde for 20 minutes, the cells were cultured with 100 μl DAPI solution (Thermo Fisher Scientific) at room temperature for 30 minutes. Then EdU-positive cells were counted applying a fluorescent microscope (Olympus, Beijing, China).

**Transwell assay**

Transwell assay was conducted to analyze cell migration and invasion. The treated cells were cultured in the top chambers (Millipore) with serum-free DMEM (Gibco) and Matrigel (BD Biosciences, USA). DMEM with 10% FBS (Gibco) was added to the bottom chambers. One day later, the invaded cells in the bottom membranes were fixed with methanol, stained with crystal violet and then counted in five randomly selected fields with an Olympus microscope. For cell migration assay, the steps were the same as those of cell invasion assay except that no Matrigel was in the top chambers.

**Luciferase reporter assay**

The pmirGLO-BTN3A1-Wt or pmirGLO-BTN3A1-Mut vectors were co-transfected with NC mimics or miR-509-3p mimics into HeLa or SiHa cells respectively, and the pmirGLO-HOXA-AS2-Wt or pmirGLO-HOXA-AS2-Mut vectors were treated as the above. Two days after transfection, the luciferase activity of the reporters was analyzed through Luciferase Reporter Assay System (Promega, USA).
RNA immunoprecipitation assay (RIP assay)

Magna RIP Kit (Millipore) was used to carry out RIP assay. The treated HeLa and SiHa cells were lysed with RIP buffer. Then the cell lysis was cultivated with magnetic beads coated with Ago2 or IgG antibodies. After RNA purification, the enrichment of RNAs was subjected to qRT-PCR assay.

Fluorescence in situ hybridization assay (FISH assay)

HeLa and SiHa cells were fastened in 4% paraformaldehyde and treated with pepsin, followed by dehydration with ethanol. Afterwards, the cells were incubated with the FISH probe (RiboBio) in hybridization solution, counterstained with DAPI and observed under an Olympus fluorescence microscope.

Tube formation assay

The frozen Matrigel was thawed at 4 °C overnight, then added to each well of prechilled 96-well plates and maintained at 37 °C for a hour. After human umbilical vein endothelial cells (HUVECs) attached to the wall, the supernatant of treated HeLa or SiHa cells was used to replace culture solution, and the cells were further cultivated for 6 hours. The right fields of view were chosen for observation and imaging with an Olympus microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism Version 5.0 (GraphPad Software, USA). Each assay was conducted at least in triplicate. Results were demonstrated as mean ± standard deviation (SD). Group differences were analyzed through Student’s t test or one-way ANOVA. Kaplan-Meier method was applied for overall survival. P < 0.05 was considered significant.

Results

HOXA-AS2 expression is obviously increased in cervical cancer

In order to evaluate the role of HOXA-AS2 in cervical cancer, we conducted qRT-PCR assay to detect the expression of HOXA-AS2 in cervical cancer at first. As a result, HOXA-AS2 exhibited higher expression in cervical cancer tissues than that in corresponding noncancerous tissues (Figure 1A). Moreover, the expression level of HOXA-AS2 in four cervical cancer cell lines (HeLa, SiHa, C33A and CaSki) was greatly elevated in comparison with that in normal human cervical epithelial cell line Ect1/E6E7 (Figure 1B). Further, as indicated by Kaplan-Meier analysis, the cervical cancer patients with high HOXA-AS2 expression had shorter overall survival time than those with low HOXA-AS2 expression (Figure 1C). Thus, we concluded that HOXA-AS2 is notably upregulated in cervical cancer tissues and cell lines, and high HOXA-AS2 expression is strongly associated with poor prognosis of cervical cancer patients.

HOXA-AS2 enhances progression and angiogenesis in cervical cancer
On the basis of the findings above, we intended to assess the critical function of HOXA-AS2 in cervical cancer process and angiogenesis. In Figure 2A, the expression of HOXA-AS2 was declined with the introduction of HOXA-AS2#1/2 vectors into HeLa and SiHa cells. Afterwards, the proliferation ability of cervical cancer cells was cut down by HOXA-AS2 knockdown (Figure 2B and 2C). Then transwell assay illustrated that the silencing of HOXA-AS2 reduced the number of migrated and invaded cells in cervical cancer, revealing that the migration and invasion capacities of cervical cancer were lessened by HOXA-AS2#1/2 vectors (Figure 2D and 2E). Besides, the number of tubes was decreased and angiogenesis-related proteins (VEGFA, Ang1 and FGF2) suffered a reduction after the deficiency of HOXA-AS2 in HeLa and SiHa cells (Figure 2F and 2G). Both outcomes uncovered that the knockdown of HOXA-AS2 inhibited angiogenesis in cervical cancer. Taken together, HOXA-AS2 contributes to progression and angiogenesis in cervical cancer.

**HOXA-AS2 sponges miR-509-3p in cervical cancer**

In the following assays, we were going to explore the regulatory mechanism of HOXA-AS2 in cervical cancer. FISH assay confirmed that HOXA-AS2 was primarily located in the cytoplasm of cervical cancer cells (Figure 3A), so HOXA-AS2 could function as a ceRNA in cervical cancer. On the condition of Pan-Cancer (8 cancer types) at starBase, only one miRNA miR-509-3p was found to harbor binding sites with HOXA-AS2, and the expression of miR-509-3p was lowly expressed in the four cervical cancer cell lines relative to that in the normal cervical epithelial cell line (Figure 3B). Then the overexpression effect of miR-509-3p was examined through qRT-PCR assay, showing that miR-509-3p expression was prominently elevated by miR-509-3p mimics (Figure 3C). What’s more, as presented by luciferase reporter assay, the luciferase activity of pmirGLO-HOXA-AS2-Wt reporters was evidently declined by miR-509-3p mimics compared with NC mimics, but that of pmirGLO-HOXA-AS2-Mut reporters displayed no remarkable change (Figure 3D). The enrichment of HOXA-AS2 and miR-509-3p in Ago2 antibodies further verified that HOXA-AS2 could bind with miR-509-3p (Figure 3E). Besides, HOXA-AS2 expression was downregulated when miR-509-3p was upregulated in cervical cancer (Figure 3F). To conclude, HOXA-AS2 absorbs miR-509-3p in cervical cancer.

**MiR-509-3p targets BTN3A1 in cervical cancer**

It has been reported that miR-509-3p targets specific mRNAs in cancers [14-16], which could also be applied to cervical cancer. With the help of Venn diagram (microT, miRmap, PITA and RNA22), five mRNAs (LARP4, GOSR1, WDR5B, BTN3A1 and IL13RA1) were identified as the candidate target genes of miR-509-3p (Figure 4A). After overexpressing miR-509-3p, BTN3A1 demonstrated the lowest expression among other four mRNAs (Figure 4B), so BTN3A1 was selected as the subject for subsequent experiments. In Figure 4C, miR-509-3p mimics repressed the luciferase activity of pmirGLO-BTN3A1-Wt reporters, while had no impact on pmirGLO-BTN3A1-Mut reporters (Figure 4C). And RIP assay elucidated that miR-509-3p and BTN3A1 were enriched in Ago2-coated beads other than in IgG-coated beads (Figure 4D). These findings implied that miR-509-3p could bind with BTN3A1. Additionally, miR-509-3p elevation or HOXA-AS2 depletion restrained BTN3A1 mRNA and its protein expressions (Figure 4E). Moreover,
BTN3A1 was highly expressed in the cervical cancer cell lines (Figure 4F). To sum up, miR-509-3p targets BTN3A1 in cervical cancer.

**HOXA-AS2 promotes cervical cancer progression and angiogenesis via miR-509-3p/BTN3A1 axis**

To validate whether HOXA-AS2 accelerated cell proliferation, migration, invasion and angiogenesis in cervical cancer by sponging miR-509-3p to modulate BTN3A1 expression, we did the exploration below. In HeLa cells, pcDNA3.1/BTN3A1 vectors raised the mRNA expression and protein level of BTN3A1 (Figure 5A). Then the upregulation of BTN3A1 counteracted HOXA-AS2 downregulation-mediated inhibition on cell proliferation in cervical cancer as manifested through MTT and EdU assays (Figure 5B and 5C). Furthermore, the restrictive effect of HOXA-AS2 silence on cell migration and invasion was reversed by BTN3A1 overexpression (Figure 5D and 5E). Besides, BTN3A1 enhancement restored the decreased number of blood vessels as well as reduced protein levels of VEGFA, Ang1 and FGF2 induced by HOXA-AS2 suppression (Figure 5F and 5G). That is, BTN3A1 overexpression partially rescued the inhibitory influence of HOXA-AS2 deficiency on cervical cancer progression and angiogenesis. Overall, HOXA-AS2 facilitates cervical cancer progression and angiogenesis via targeting miR-509-3p/BTN3A1 axis.

**Discussion**

Cervical cancer has a serious impact on public health, and HOXA-AS2 may be helpful for cervical cancer treatment. A growing body of evidence has suggested that HOXA-AS2 functions as a tumor-promoter in the biological activities of diverse cancers, such as breast cancer, bladder cancer and papillary thyroid cancer [11-13]. But the biological role of HOXA-AS2 in cervical cancer awaits illumination. This study firstly revealed that HOXA-AS2 expression was distinctly elevated in cervical cancer tissues and cell lines, and high HOXA-AS2 expression predicted poor prognosis of cervical cancer patients. Further, HOXA-AS2 accelerated cell proliferation, migration, invasion and angiogenesis in cervical cancer.

It’s worth noting that as competing endogenous RNAs (ceRNAs), lncRNAs affect the expression of target genes through sponging miRNAs in the progress of cancers [17]. MicroRNAs (miRNAs) are a type of short non-coding RNAs with 21-25 nucleotides, acting as key players in the regulation of numerous tumors [18]. For example, miRNA-493-5p cuts down the expression of MYCN to repress cell growth and invasion in hepatic cancer [19]. MiR-324-5p inhibits cell metastasis in gallbladder carcinoma via downregulating TGFB2 [20]. What’s more, miRNAs also take part in the aggressive behaviors of cervical cancer. For instance, miR-145 fosters the progression of cervical cancer by targeting FSCN1 [21]. MiR-204 affects cell proliferation, apoptosis and autophagy in cervical cancer by modulating ATF2 [22]. In present exploration, we speculated that in cervical cancer, HOXA-AS2 could combine with miR-509-3p which inhibits the development of cancers. For instance, miR-509-3p suppresses cellular migration, invasion, proliferation and cisplatin sensitivity of osteosarcoma [23]. MiR-509-3p impedes cell proliferation and migration via targeting XIAP in gastric cancer [14]. MiR-509-3p restrains cell proliferation and migration by combining with MAP3K8 in renal cell carcinoma [15]. Then through our validation, we concluded that HOXA-AS2
acted as a sponge of miR-509-3p and negatively regulated the expression of miR-509-3p in cervical cancer.

As ceRNA networks present, miRNAs can modulate gene or protein expression through binding with mRNAs [24]. And miR-509-3p was found to have binding sites with mRNA butyrophilin 3A1 (BTN3A1). BTN3A1 is closely linked to the overall survival of bladder cancer patients [25]. BTN3A1 regulates the development of colorectal cancer [26]. BTN3A1 owns great prognostic significance in patients with pancreatic cancer [27]. Further, our investigation unfolded that BTN3A1 was a downstream target of miR-509-3p in cervical cancer. Additionally, rescue assays confirmed that BTN3A1 enhancement partially offset HOXA-AS2 deficiency-induced inhibition on cervical cancer progression and angiogenesis.

Collectively, the paper delineated that HOXA-AS2 facilitated cell proliferation, migration, invasion and angiogenesis in cervical cancer by sponging miR-509-3p to regulate BTN3A1 expression. In short, HOXA-AS2/miR-509-3p/BTN3A1 axis greatly influenced cervical cancer progression and angiogenesis, which may be an innovative glimpse into therapeutic methods of cervical cancer.

**Abbreviations**

IncRNAs: long non-coding RNAs

HOXA-AS2: IncRNA HOXA cluster antisense RNA 2

ATCC : American Type Culture Collection

FBS: fetal bovine serum

DMEM: Dulbecco’s modified Eagle’s medium

qRT-PCR: quantitative real-time polymerase chain reaction

cDNA: complementary DNA

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF: polyvinylidene difluoride

MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide

DMSO: dimethyl sulphoxide

OD: optical density

EdU: 5-ethynyl-2'-deoxyuridine

RIP assay: RNA immunoprecipitation assay
FISH assay: Fluorescence in situ hybridization assay

HUVECs: human umbilical vein endothelial cells

SD: standard deviation

ceRNAs: competing endogenous RNAs

miRNAs: MicroRNAs

BTN3A1: butyrophilin 3A1

**Declarations**

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center.

**Availability of data and material**

The datasets used during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that no conflict of interests exists.

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**Authors’ contributions**

Both two co-authors participated in the literature search, analysis and interpretation of the data, and the writing of the manuscript. They saw and approved the final manuscript.

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**Figures**

![Figure 1](image_url)

**Figure 1**

HOXA-AS2 expression is obviously increased in cervical cancer. (A) The expression of HOXA-AS2 in cervical cancer tissues and adjacent normal tissues was detected by qRT-PCR assay. *P* < 0.05 vs. Normal group. (B) HOXA-AS2 expression in four cervical cancer cell lines (HeLa, SiHa, C33A and CaSki) and one normal human cervical epithelial cell line Ect1/E6E7 was subjected to qRT-PCR assay. *P* < 0.05 vs. Ect1/E6E7 group. (C) The impact of HOXA-AS2 expression on the prognosis of cervical cancer patients was analyzed by Kaplan-Meier analysis.
Figure 2

HOXA-AS2 enhances progression and angiogenesis in cervical cancer. (A) The knockdown efficiency of HOXA-AS2 in cervical cancer cells was tested via qRT-PCR assay. *P < 0.05 vs. sh-NC group. (B-C) Cell proliferation was assessed by MTT and EdU assays. *P < 0.05 vs. sh-NC group. (D-E) Cell migration and invasion were measured through transwell assay. *P < 0.05 vs. sh-NC group. (F) The angiogenesis capacity of HUVECs was evaluated by tube formation assay. *P < 0.05 vs. sh-NC group. (G) The levels of angiogenesis-associated proteins (VEGFA, Ang1 and FGF2) were detected by western blot assay.
Figure 3

HOXA-AS2 sponges miR-509-3p in cervical cancer. (A) The location of HOXA-AS2 in cervical cancer cells was confirmed by FISH assay. (B) One HOXA-AS2-binding miRNA was discovered via starBase website, and qRT-PCR assay was used to examine its expression in cervical cancer cell lines. P < 0.05 vs. Ect1/E6E7 group. (C) The overexpression effect of miR-509-3p in cervical cancer cells was detected through qRT-PCR assay. P < 0.05 vs. NC mimics group. (D) The interaction between miR-509-3p and HOXA-AS2 was verified by luciferase reporter assay. P < 0.05 vs. NC mimics group. (E) RIP assay was applied to determine the interaction between miR-509-3p and HOXA-AS2. P < 0.05 vs. IgG group. (F) The
influence of miR-509-3p on HOXA-AS2 expression was assessed via qRT-PCR assay. P < 0.05 vs. NC mimics group.
BTN3A1-Wt: 5’ cccUCCACAG-C-AACCAUCA 3’
miR-509-3p: 3’ gauGGUUGCUCAUUGUAU6a 5’
BTN3A1-Mut: 5’ cccGGUUGC-G-AUGGUA6a 3’
Figure 4

MiR-509-3p targets BTN3A1 in cervical cancer. (A) MiR-509-3p-binding mRNAs were predicted by starBase website. (B) The expressions of the mRNAs in treated cells were measured via qRT-PCR assay. P < 0.05 vs. NC mimics group. (C) Luciferase reporter assay was performed to validate the interaction between miR-509-3p and BTN3A1. P < 0.05 vs. NC mimics group. (D) The interaction of miR-509-3p and BTN3A1 was tasted via RIP assay. P < 0.05 vs. IgG group. (E) Western blot and qRT-PCR assays were utilized to evaluate the influences of miR-509-3p and HOXA-AS2 on BTN3A1 mRNA and its protein expressions. P < 0.05 vs. NC mimics group or sh-NC group. (F) The expression of BTN3A1 in cervical cancer cell lines was detected by qRT-PCR assay. P < 0.05 vs. Ect1/E6E7 group.
HOXA-AS2 promotes cervical cancer progression and angiogenesis via miR-509-3p/BTN3A1 axis. (A) The overexpression efficiency of BTN3A1 in cervical cancer cells was tested through qRT-PCR assay. P < 0.05 vs. pcDNA3.1 group. (B-C) MTT and EdU assays were conducted to detect cell proliferation. P < 0.05. (D-E) Transwell assay was applied to assess cell migration and invasion. P < 0.05. (F) The angiogenesis ability of HUVECs was measured by tube formation assay. P < 0.05. (G) Western blot assay was performed to probe the protein levels of VEGFA, Ang1 and FGF2.