Long non-coding RNA DLX6-AS1 promotes proliferation by acting as a ceRNA targeting miR-199a in cervical cancer

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Abstract. Emerging evidence has revealed significant roles for long noncoding RNA (lncRNA) in various biological processes, including cell proliferation, apoptosis and invasion. The lncRNA distal-less homeobox 6 antisense 1 (DLX6-AS1) has been reported to serve as a vital oncogene during tumorigenesis and progression. However, the expression levels and functional roles of DLX6-AS1 in cervical cancer are not yet well understood. In the present study, DLX6-AS1 expression was identified to be significantly upregulated in cervical cancer tissues and cell lines by reverse transcription-quantitative polymerase chain reactions. Knockdown of DLX6-AS1 inhibited cell proliferation and induced cell apoptosis. Bioinformatics analysis predicted that micro RNA (miR)-199a was a direct target of DLX6-AS1. Overexpression of miR-199a counteracted the role of DLX6-AS1 in facilitating proliferation and inhibiting apoptosis in *in vitro* rescue assays. The present results suggest that DLX6-AS1 acting as a sponge for miR-199a may serve a critical role in the development and progression of cervical cancer.

Introduction

Cervical cancer, the second most predominant cancer in females, is the fourth most fatal cancer in women worldwide (1). Human papillomavirus (HPV) has been demonstrated to cause the initiation and progression of ~99% of cervical tumors (2). Conventional treatment of cervical cancer, including surgery, chemotherapy and radiotherapy, may significantly increase chances of survival (3,4). In total, ~90% of novel cases occurring in developing countries may be due to the lack of nationwide screening programs and HPV vaccines (3,4). However, the pathogenesis and exact molecular mechanisms underlying the progression of cervical cancer require further investigation. Therefore, the identification of alterations to crucial genes associated with cervical cancer is urgently required.

Long non-coding RNAs (lncRNAs), a class of endogenous non-coding RNAs, are >200 nucleotides in length and lack protein-coding capacity (5,6). Accumulating evidence has suggested that lncRNAs serve a vital regulatory role in numerous physiological and pathological processes, including cell proliferation, apoptosis, migration, invasion, differentiation and angiogenesis (5-8). Aberrant expression of lncRNAs is involved in the progression of a variety of diseases, including cervical cancer (5,9,10). For example, downregulated XLOC_010588 expression is an independent poor prognostic factor and may serve as a predictor of poor prognosis for patients with cervical cancer (11). Knockdown of XLOC_010588 inhibits cervical cancer cell growth by decreasing the expression of proto-oncogene c-Myc (11). Overexpression of the lncRNA HOTAIR promoted cervical cancer cell proliferation, migration and invasion and was associated with lymph node metastasis and shorter overall survival (12). lncRNA MEG3 expression level is significantly lower in cervical cancer tissues, and downregulation of MEG3 expression is correlated with the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage and lymph node metastasis in cervical cancer patients (10). lncRNA DLX6-AS1 expression is significantly higher in lung adenocarcinoma tissues and high DLX6-AS1 expression is correlated with higher histological differentiation and advanced Tumor, Node and Metastasis (TNM) stages (13). Furthermore, DLX6-AS1 modulates cell growth and tumorigenesis via regulation of the micro RNA (miRNA/miR)-26a/PTEN axis in renal cell carcinoma (14). In addition, DLX6-AS1 silencing inhibits the proliferation, migration and invasion of hepatocellular carcinoma cells via the miR-203a/matrix metalloprotease (MMP)-2 pathway (15). However, the expression and biological functions of DLX6-AS1 in cervical cancer remain largely unknown.

In the present study, it was observed that DLX6-AS1 expression was significantly increased in cervical cancer tissues and cell lines. Furthermore, DLX6-AS1 knockdown

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impaired cell proliferation and induced cell apoptosis *in vitro*. The interaction between DLX6-AS1 and miR-199a was additionally examined to reveal the underlying mechanism of DLX6-AS1 in cervical cancer.

Materials and methods

Tissue samples. A total of 78 paired adjacent noncancerous specimens and cervical cancer samples were collected from patients who had undergone surgical resection at the Department of Gynecology of the Women's Hospital of Zhejiang University (Hangzhou, China) between May 2010 and December 2013. Patients (age range, 37-62 years, and mean 52.4 years) were diagnosed with cervical cancer by histological pathology. Tumor grades and stages were adjusted to comply with the new 2009 classification (16). None of the patients had received preoperative chemotherapy or radiotherapy. All patients enrolled in the present study were pathologically confirmed as having cervical cancer, and written informed consent was obtained from each patient. The present study was approved by the Ethics Committee of Zhejiang University (license no. 2016-01AH). All samples were frozen at the time of surgery in liquid nitrogen and stored at -80˚C until use.

Cell lines. Human immortalized cervical epithelial cell lines (NC104) and human cervical cancer cell lines (CaSki, ME-180, C-33A, SiHa and HeLa) were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml), and streptomycin (100 mg/ml) in a humidified incubator at 37˚C in 5% CO_2.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissues and cells was isolated using Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was reverse transcribed to complementary DNA (cDNA) using PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) under conditions of 30˚C for 10 min, 42˚C for 30 min, 95˚C for 5 sec, and 4˚C for 10 min. RT-qPCR was performed using Syber Green PCR mastermix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The sequences of the primers were as follows: lncRNA DLX6-AS1, 5'-AGTTTCTCTCTAGATTGC CTT-3' (forward) and 5'-ATTGACATGTTAGTGCCCTT-3' (reverse); and GAPDH, 5'-GCACCGTCAAGGCTGAGA AC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse). PCR was performed under the following parameters: Pre-denaturation cycle of 2 min at 94˚C, 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, and 72˚C for 2 min, with a final extension at 72˚C for 5 min. GAPDH was used as a loading control. The relative expressions of DLX6-AS1 were normalized to GAPDH and calculated according to $2^{-\Delta\Delta Cq}$ method (17).

Cell transfection. Specific small interfering (si)RNA oligonucleotides (Shanghai GenePharma Co., Ltd., Shanghai, China) targeting DLX6-AS1 and negative control siRNAs were obtained from Shanghai GenePharma Co., Ltd. miR-199a mimics, miR-199a inhibitor and its negative control were additionally purchased from Shanghai GenePharma Co., Ltd. For transfection, a total of $6x10^5$ cells were seeded into 100 mm culture dishes and transfected with the respective RNA oligonucleotides at a final concentration of 50 nm, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol.

Cell viability and proliferation assay. Cells transfected with a corresponding oligonucleotide were seeded into 96-well plates at a density of $3x10^4$ cells/well. A volume of 10 μl Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well at 24, 48, 72 and 96 h of incubation, followed by an incubation of 2 h. The absorbance at 450 nm was measured using a SpectraMaxM3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Colony formation assay. The cells were seeded in each well of a 6-well culture plate at a density of 600 cells/well and cultured in DMEM supplemented with 10% FBS for 2 weeks with the growth media replaced every third day. The colonies (containing ≥50 cells) were washed with PBS, fixed with methanol for 15 min at room temperature and stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 15 min at room temperature. Cell number was calculated under an inverted light microscope (TS100; Nikon Corporation, Tokyo, Japan).

Cell apoptosis analyses. Cell apoptosis was assessed with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Cells were harvested and washed twice with PBS, and 5 μl PI and 5 μl FITC were added and incubated at room temperature in the dark for 15 min. Cell samples were analyzed using a flow cytometer (FACScan; BD Biosciences) with CellQuest Pro Software (version 5.1, BD Biosciences).

Reporter vectors construction and luciferase assays. To identify miRNAs that bind DLX6-AS1, predictions were made with starBase v2.0 (starbase.sysu.edu.cn/mirLncRNA.php). The sequence containing the mir-199a binding sites plus 500 base pairs at 5'- and 3'-flanking regions in DLX6-AS1 was amplified by PCR using PrimeSTAR® Max DNA Polymerase (Takara Biotechnology Co., Ltd.) and cloned into Psi-CHECK-2 vectors (Promega Corporation, Madison, WI, USA). The sequence of primers as follows: (forward) 5'-GGCCTAGTCAACCAA GGATG-3' and (reverse) 5'-GGTCCCCTTGCTGAAGAT TAC-3'. The corresponding mutants were created by mutating the mir-199a seed-region binding site using QuikChange® Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA). A total of 1,000 cervical cancer cells were plated in a 96-well plate (Corning Incorporated, Corning, NY, USA) and co-transfected with 100 ng wild-type Psi-CHECK-2-DLX6-AS1 (or DLX6-AS1 mutant) reporter

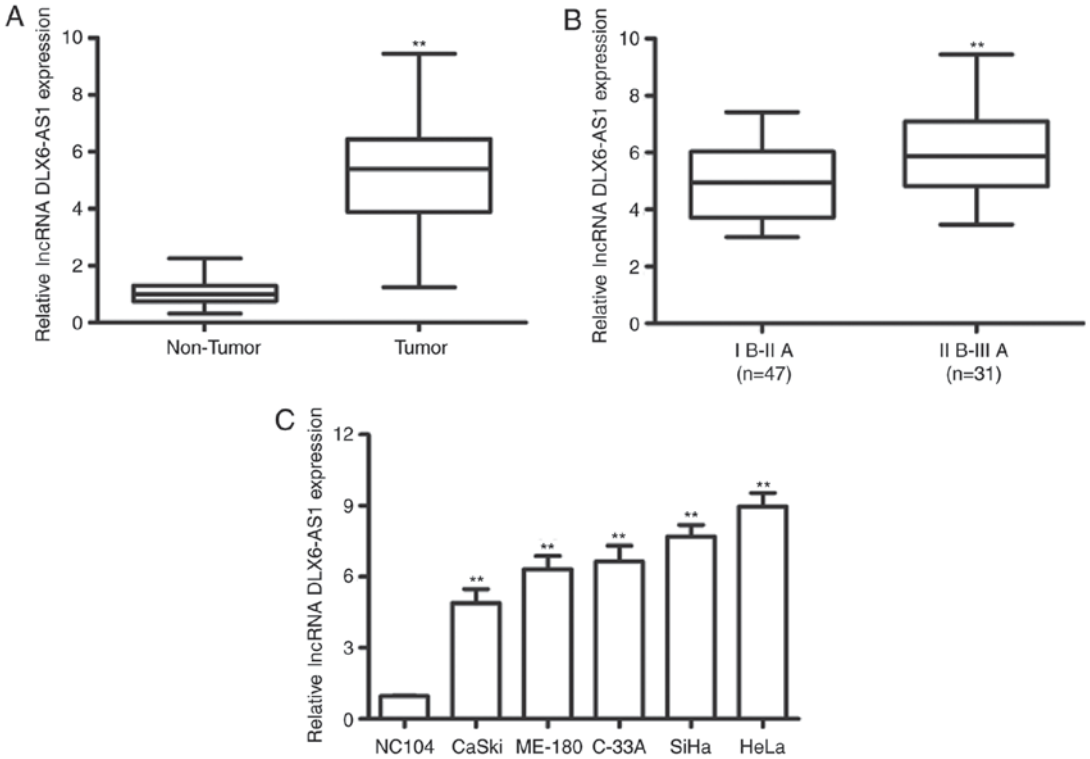

Figure 1. lncRNA DLX6-AS1 is significantly upregulated in cervical cancer tissues and cell lines. (A) Relative expression of DLX6-AS1 in 78 pairs of cervical cancer tissue and matched non-tumor tissues samples by RT-qPCR analysis. **P<0.01 vs. non-tumor. (B) DLX6-AS1 was highly expressed in cervical cancer in advanced Fédération Internationale de Gynécologie et d'Obstétrique stages. **P<0.01 vs. I B-II A. (C) Expression levels of DLX6-AS1 were determined by RT-qPCR in immortalized cervical epithelial cell lines and various cervical cancer cells. **P<0.01 vs. NC104. lnc, long non-coding; DLX6-AS1, distal-less homeobox 6 antisense 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

plasmid and 20 ng miR-199a mimics or mimic controls using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The luciferase activities were measured using the Dual-Luciferase Reporter Assay System and normalized to Renilla luciferase activities (Promega Corporation) after 36 h, according to the manufacturer's protocol.

Statistical analysis. Data are expressed as the mean ± standard deviation, and statistical analysis was performed using GraphPad Prism (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). These experiments were performed in triplicate. Differences between groups were tested using paired and unpaired Student's t-test, and one-way analysis of variance followed by Tukey's post hoc test were performed to assess the difference between more than two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA DLX6-AS1 expression is upregulated in cervical cancer tissues and cells. Altered DLX6-AS1 expression has been identified in a number of types of tumors; however, whether aberrant DLX6-AS1 expression is involved in cervical cancer development remains unknown. Expression levels of DLX6-AS1 were investigated in the 78 cervical cancer tissue samples and matched non-tumor adjacent tissue samples using RT-qPCR. As demonstrated in Fig. 1A, DLX6-AS1 was significantly overexpressed in cervical cancer tissue samples compared with the non-tumor tissue samples from the same patient (P<0.01). Furthermore, DLX6-AS1 expression was significantly increased in patients at advanced FIGO stages (II B-III A phase) compared with those at earlier clinical stages (I B-II A phase; P<0.01; Fig. 1B). In addition, the expression of DLX6-AS1 in five human cervical cancer cell lines (CaSki, ME-180, C-33A, SiHa and HeLa) and immortalized cervical epithelial cell lines (NC104) was examined. The expression of DLX6-AS1 was significantly upregulated in cervical cancer cells compared with the NC104 cells (P<0.01; Fig. 1C). These results suggested that DLX6-AS1 may serve as an oncogene involved in the tumorigenesis and progression of cervical cancer.

lncRNA DLX6-AS1 affects cervical cancer cell proliferation. To investigate the biological role of DLX6-AS1 in the progression of cervical cancer, DLX6-AS1 was knocked down in SiHa and HeLa cells with high endogenous DLX6-AS1 expression by transfecting with specific DLX6-AS1 siRNAs. As demonstrated in Fig. 2A, transfection with siRNA#1 and siRNA#2 significantly decreased the DLX6-AS1 expression levels in SiHa and HeLa cells compared with the negative control (NC; P<0.01). siRNA#2 was more effective at inhibiting DLX6-AS1 expression; therefore, this construct was termed 'siRNA' and used for all subsequent experiments. A CCK-8 assay demonstrated that DLX6-AS1 silencing in SiHa and HeLa cell lines resulted in a decrease in proliferative capability compared with the NC (Fig. 2B). Subsequently, apoptosis was assessed using flow cytometry, and the results demonstrated that silencing DLX6-AS1 led to a significant increase in the apoptotic rate

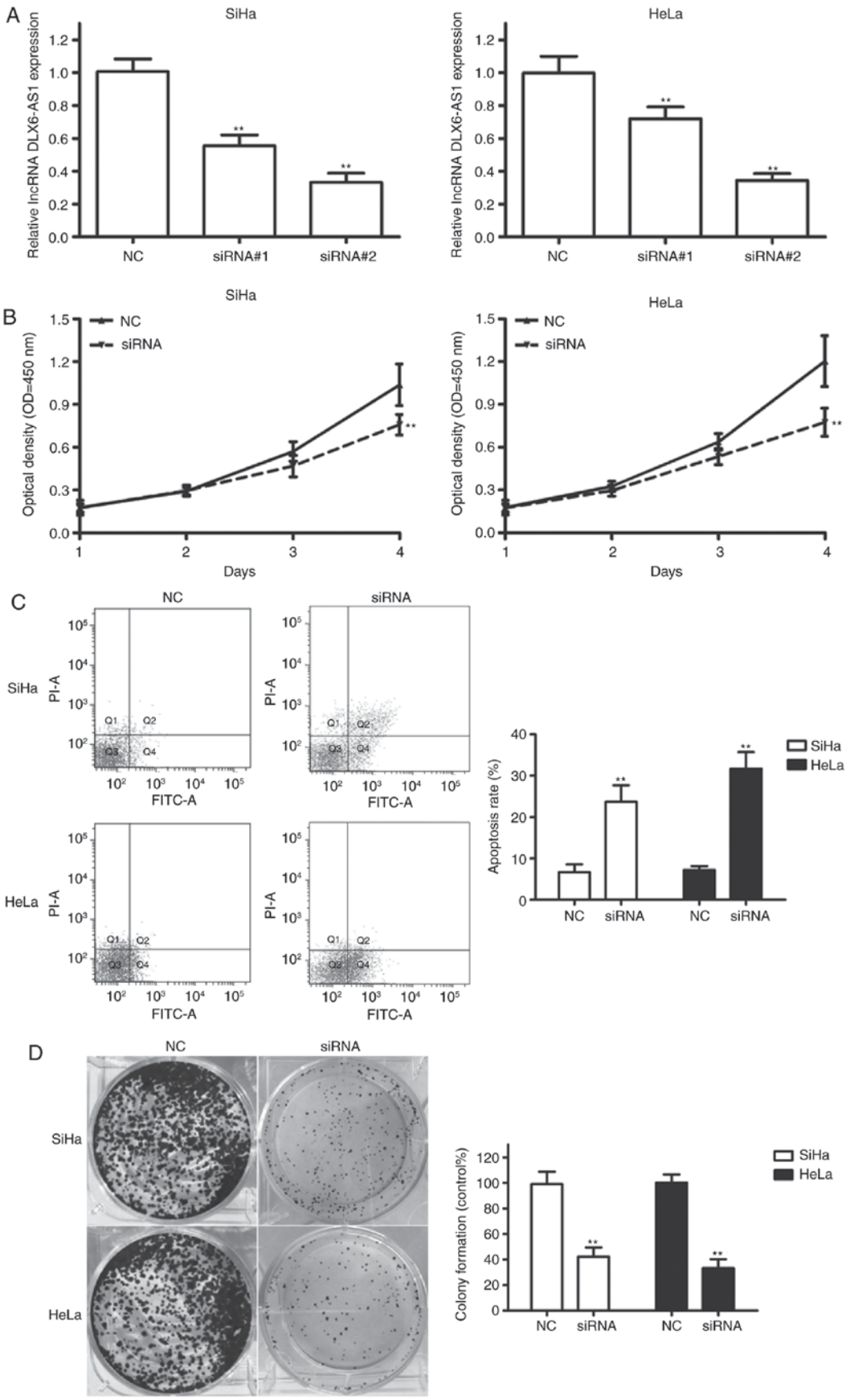

Figure 2. Knockdown of DLX6-AS1 inhibits cell proliferation and promotes cell apoptosis. (A) Transfection efficiency of SiHa and HeLa cells transfected with special siRNAs or corresponding NC was verified by reverse transcription-quantitative polymerase chain reaction. (B) Cell Counting Kit-8 assays were performed to evaluate proliferation. (C) Colony formation assays demonstrate the clone number in cervical cancer cells transfected with DLX6-AS1 siRNAs or NC. (D) Cell apoptosis was assessed by flow cytometry. **P<0.01 vs. respective NC. lnc, long non-coding; DLX6-AS1, distal-less homeobox 6 antisense 1; si, small interfering; NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate; A, Annexin V.

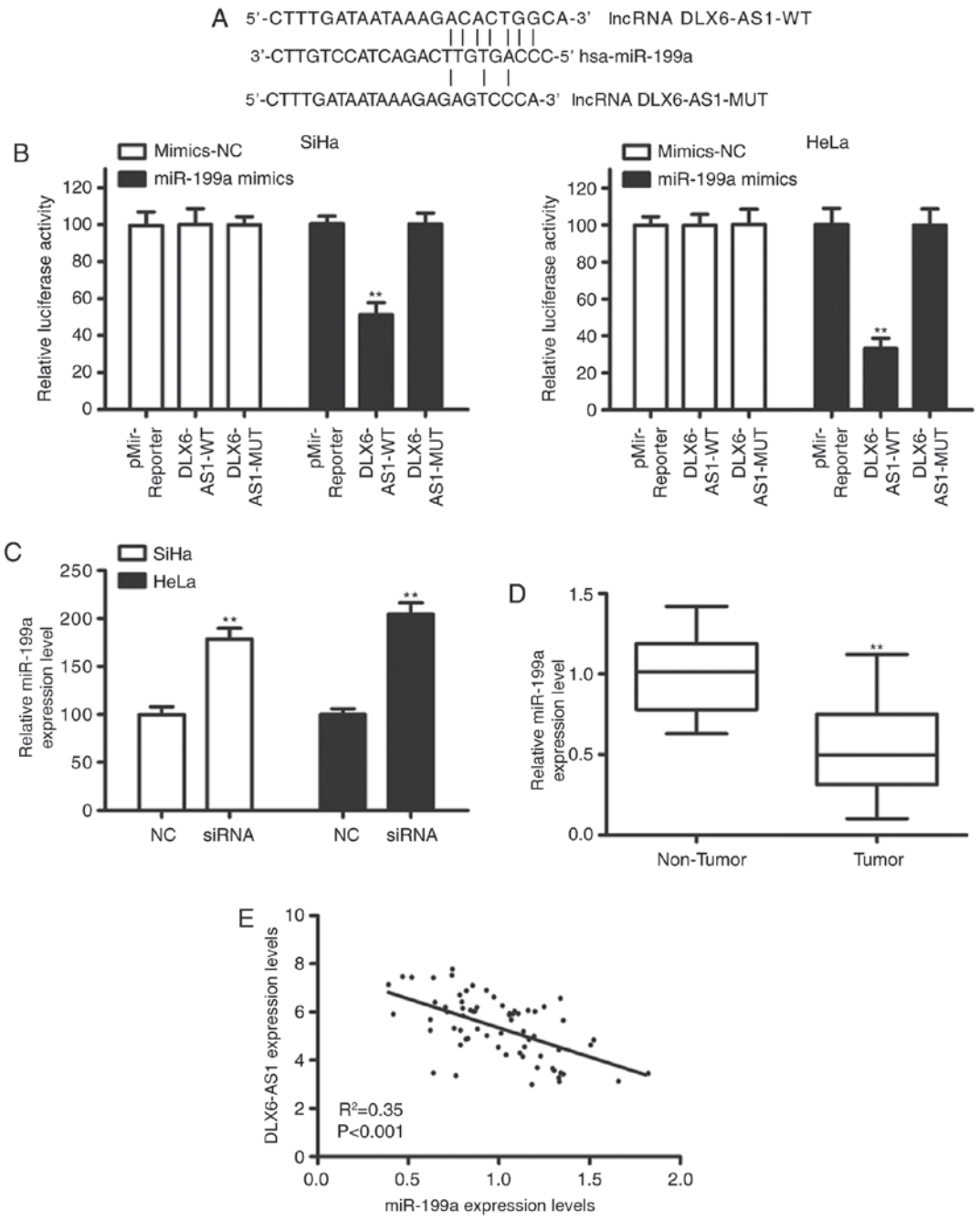

Figure 3. DLX6-AS1 acts as a sponge for miR-199a. (A) Schematic representation of the binding sites between the DLX6-AS1 and miR-199a. (B) Luciferase activity was measured by co-transfection of a miR-199a mimic and a luciferase reporter plasmid in cervical cancer cells. **P<0.01 vs. pMir-Reporter. (C) miR-199a is increased in DLX6-AS1-silenced cells compared with NC. **P<0.01 vs. NC. (D) Expression of miR-199a in 78 pairs of cervical cancer tissue and matched non-tumor tissues. **P<0.01 vs. non-tumor. (E) Lower miR-199a expression in cervical cancer tissues is negatively correlated with higher DLX6-AS1 expression by Spearman correlation coefficient analysis. DLX6-AS1, distal-less homeobox 6 antisense 1; miR, micro RNA; NC, negative control; lnc, long non-coding; WT, wild-type; MUT, mutant; si, small interfering.

of the two cell lines compared with the NC (Fig. 2C; P<0.01). In addition, colony-formation assays revealed a significant decrease in colony formation following DLX6-AS1 knockdown in the cervical cancer cell lines (Fig. 2D; P<0.01). Therefore, it may be concluded that DLX6-AS1 mediates cervical cancer cell proliferation by regulating cell apoptosis.

Reciprocal repression exists between lncRNA DLX6-AS1 and miR-199a in cervical cancer cells. Increasing evidence has demonstrated that lncRNAs may serve as competitive endogenous RNAs (ceRNAs) to regulate the target mRNAs of miRNAs (6). A number of miRNAs were predicted to target DLX6-AS1 (data not shown); however, miR-199a was selected as miR-199a is reported to serve as a tumor suppressor (16-18). The binding sites of DLX6-AS1 and miR-199a are presented in Fig. 3A. Dual reporter luciferase assays revealed that co-transfection of miR-199a mimics and DLX6-AS1-wild-type (WT) luciferase reporter constructs significantly reduced the luciferase activity of DLX6-AS1-WT in SiHa and HeLa cell lines (P<0.01; Fig. 3B).

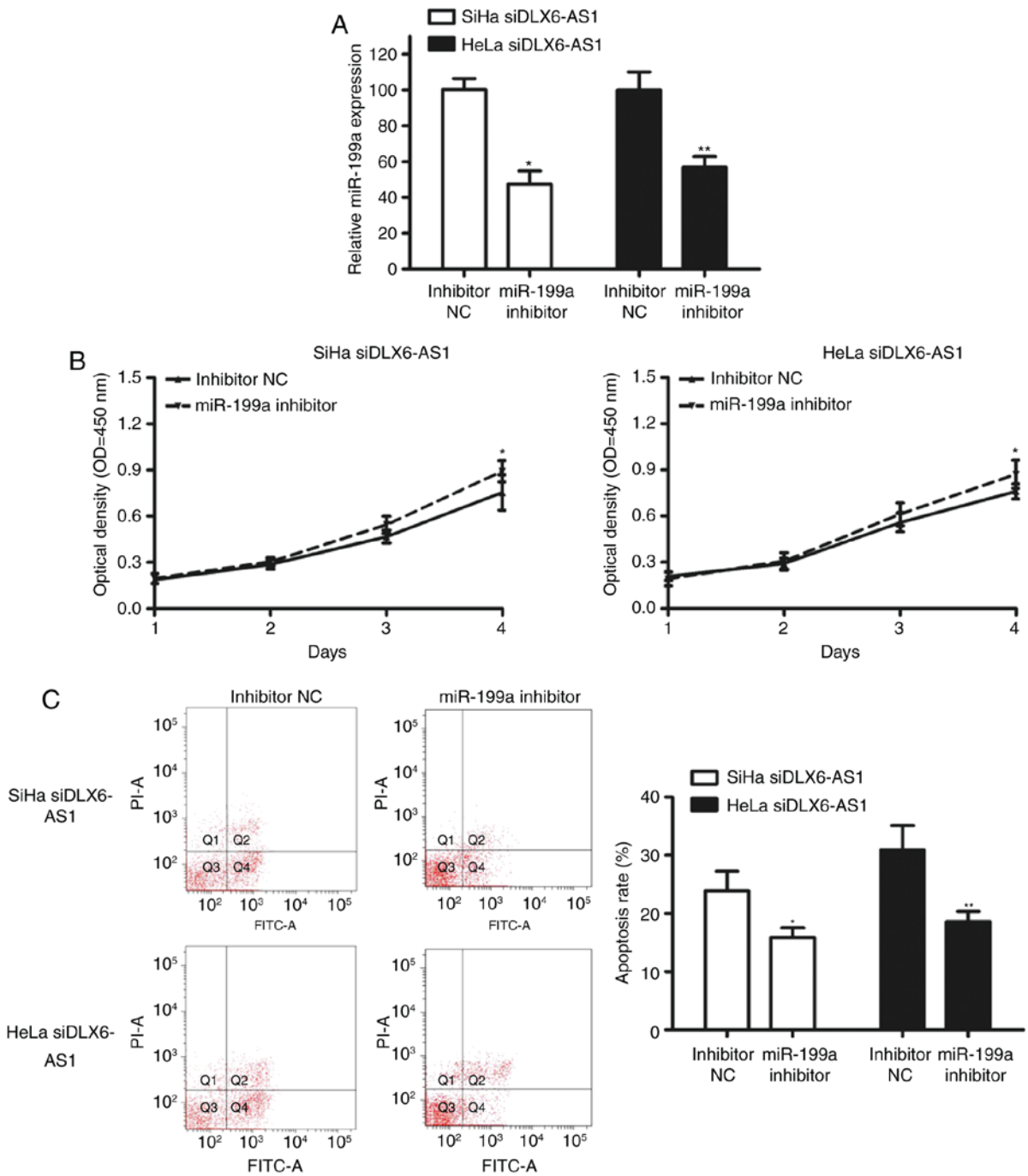

Figure 4. miR-199a mediates the effects of DLX6-AS1 on cervical cancer cells. (A) Expression of miR-199a was detected in DLX6-AS1-silenced cells transfected with miR-199a inhibitor or inhibitor NC. (B) Cell proliferation and (C) apoptosis were measured in DLX6-AS1 deletion cervical cancer cells transfected with miR-199a inhibitor or inhibitor NC. *P<0.05, **P<0.01 vs. inhibitor NC. miR, micro RNA; DLX6-AS1, distal-less homeobox 6 antisense 1; NC, negative control; si, small interfering; PI, propidium iodide; FITC, fluorescein isothiocyanate; A, Annexin-V.

However, this inhibition was eliminated by the introduction of nucleotide alterations to the predicted seed-binding sequences of miR-199a (Fig. 3B). Furthermore, knockdown DLX6-AS1increased miR-199a expression in cervical cancer cells (Fig. 3C). The expression of miR-199a in clinical samples was assessed and it was observed that the expression of miR-199a in cervical cancer tissues was significantly decreased compared with adjacent normal tissue (P<0.01; Fig. 3D). Spearman correlation analysis demonstrated a significant inverse correlation between miR-199a expression and DLX6-AS1 expression (Fig. 3E). These results suggested that DLX6-AS1 may serve as an oncogene by medicating miR-199a expression in cervical cancer.

DLX6-AS1 enhances cervical cancer cell proliferation and migration by inhibiting miR-199a expression. To determine whether DLX6-AS1 mediates decreased miR-199a-induced inhibition of proliferation and cell apoptosis *in vitro*, miR-199a was inhibited in DLX6-AS1 knockdown cells by transfection with miR-199a inhibitors, which resulted in a significant decrease of miR-199a in SiHa and HeLa cells (Fig. 4A). As hypothesized, miR-199a silencing led to a marked higher cell

proliferation rate (Fig. 4B) and a significantly lower apoptosis rate (Fig. 4C) compared with the control cells, which indicated that DLX6-AS1 partly functions by downregulating miR-199a.

Discussion

An increased understanding of the important role of non-coding RNA in the initiation and progression of multiple cancer types has revealed an exciting avenue for the development of novel cancer therapies (5,9,19). Until now, the molecular mechanism and effect of lncRNAs were largely unknown in cervical cancer tumorigenesis and progression. In the present study, the expression and molecular mechanisms of DLX6-AS1 in cervical cancer were investigated. High expression of DLX6-AS1 was observed in cervical cancer tissues and cells, and expression increased with the FIGO stage. Furthermore, knockdown of DLX6-AS1 using siRNA inhibited cell proliferation and induced apoptosis *in vitro*. In addition, the present study provided evidence that DLX6-AS1 exerted oncogene functions by downregulating miR-199a, a well-known tumor suppressor.

A previous study has suggested that DLX6-AS1 acts as an oncogene in lung adenocarcinoma and that high DLX6-AS1 expression levels are significantly associated with histological differentiation and TNM stage (13). It has been recently demonstrated that DLX6-AS1 is upregulated in renal cell carcinoma and that DLX6-AS1 promotes renal cell carcinoma cell growth and tumorigenesis by functioning as a ceRNA to sponge miR-26a (14). Furthermore, DLX6-AS1 promotes cell proliferation, migration and invasion by regulating the miR-203a/MMP-2 pathway (15). Consistent with these results, DLX6-AS1 was additionally identified in cervical cancer tissues and cell lines, and the expression of DLX6-AS1 was positively associated with FIGO stage. DLX6-AS1 knockdown decreased the proliferation of cervical cancer cells by inducing cell apoptosis.

A previous study suggested that lncRNAs serve as ceRNAs to sponge miRNAs and regulate the biological function of cancer cells (19). To elucidate the mechanism of DLX6-AS1 in cervical cancer, bioinformatics analysis was performed to predict miRNAs that bind DLX6-AS1. Notably, it was confirmed that expression levels of miR-199a were downregulated in cervical cancer tissues. Furthermore, it was observed that the expression level of DLX6-AS1 was negatively correlated with the expression level of miR-199a in cervical cancer samples. Knockdown of miR-199a abrogated the inhibition of cell growth and apoptosis caused by DLX6-AS1, which was consistent with previous studies of cancer cells (14,15,20,21), suggesting that DLX6-AS1 may promote cell proliferation by serving as a ceRNA to sponge miR-199a in cervical cancer.

In conclusion, the present results demonstrated that DLX6-AS1 expression was higher in cervical cancer, and high DLX6-AS1 expression was associated with FIGO stage in patients with cervical cancer. Furthermore, knockdown of DLX6-AS1 significantly induced cell apoptosis and inhibited cell growth and as an inverse correlation was identified between miR-199a and DLX6-AS1 expression, so knockdown of DLX6-AS1 results in an increased miR-199a expression. These results indicate that DLX6-AS1 is important for cervical cancer progression and suggest that it may be used as a potential therapeutic target for cervical cancer treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XW and YL contributed to design, wrote, and revised the manuscript. JL collected and classified the human BC tissue samples. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zhejiang University (license no. 2016-01AH) and written informed consent was obtained from each patient.

Patient consent for publication

Written informed consent was obtained from all human subjects.

Competing interests

The authors declare that they have no competing interests.

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