Long non-coding RNA AK001903 regulates tumor progression in cervical cancer

GUANGLEI ZHONG[1,2*], XINGYU FANG[1,2*], QINGSHENG XIE[1], YUHAN WANG[1], ZHONGQIU LIN[1], RONGCHUN LIN[1] and TINGTING YAO[1,2]

[1]Department of Gynecological Oncology; [2]Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510120, P.R. China

Received May 6, 2020; Accepted November 2, 2020

DOI: 10.3892/ol.2020.12338

Abstract. A majority of cervical cancers are squamous cell carcinomas, arising from the squamous (flattened) epithelial cells that line the cervix. Long noncoding RNAs (lncRNAs) are a unique class of messenger RNA-like transcripts of at least 200 nucleotides in length with no significant protein-coding capacity. Aberrant lncRNA expression is emerging as a major component of the cancer transcriptome. In the present study, lncRNA microarrays were conducted to investigate the differentially expression lncRNAs in cervical cancer (CC) tissues compared with peritumoral tissues. Then, the most significantly upregulated lncRNA, which was lncRNA-AK001903 was selected to conduct further experiments. Real-time Quantitative polymerase chain reaction was conducted to investigate lncRNA-AK001903 expression in CC tissues and Hela, Siha, Ca Ski, C33a, H8 (HPV-immortalized cervical epithelial cell line) cell lines, and *in situ* hybridization histochemistry (ISHH) was performed to detect lncRNA-AK001903 expression level in different CC stages. The effect of lncRNA-AK001903 on cell proliferation, invasion and migration was assessed after knockdown of lncRNA-AK001903. The findings of the study confirmed that lncRNA-AK001903 was upregulated in CC cells and tissues compared with normal cell line H8 and peritumoral tissues. ISHH demonstrated that the expression level of lncRNA-AK001903 was connected with International Federation of Gynecology and Obstetrics (2018) stage of CC. Knockdown of lncRNA-AK001903 inhibited cell proliferation, invasion and migration in Ca Ski cells. In conclusion, lncRNA-AK001903 was demonstrated to be an oncogenic lncRNA that promotes tumor progression and may be an effective target for CC treatment in the near future.

Correspondence to: Professor Tingting Yao or Dr Rongchun Lin, Department of Gynecological Oncology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yan Jiang West Road, Guangzhou, Guangdong 510120, P.R. China
E-mail: yaotting@mail.sysu.edu.cn
E-mail: linrchun@mail.sysu.edu.cn

*Contributed equally

Key words: microarray, long non-coding RNAs, cervical cancer, long non-coding RNA AK001903, proliferation

Introduction

Cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women, with an estimated 570,000 cases and 311,000 deaths having occurred worldwide in 2018 (1). Persistent human papilloma virus (HPV) infection is a direct cause for CC (2). With the development of DNA examination and cytological screening for high-risk HPV and the promotion of the cervical cancer vaccine, the morbidity rate of cervical cancer has been declining (3). However, the vaccines only cover some of the cancer-causing ('high-risk') types of HPV, such as HPV-16 and HPV-18 (4). Women should do regular Pap smear screening even after vaccination (5). Inspite of advances in the treatment of CC, the 5-year survival rate remains <50% in China (6). Hence, it is particularly necessary to find a new and effective treatment strategy targeting CC.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs with >200 nucleotides and are involved in various biological activities including cell proliferation, migration, invasion, apoptosis and inflammatory responses (7,8). LncRNA is a kind of non-coding RNA which modulates gene expression through epigenetic control, transcriptional regulation, and post-transcriptional regulation (9). Multiple reports have verified that abnormal expression of lncRNAs could affect the expression or function of oncogenes, tumor suppressors, metabolic enzyme genes as well as transcription factors and signaling pathways (10-14). For example, enforced expression of HOX transcript antisense RNA (HOTAIR) increased invasiveness and metastasis in breast cancer (11). Maternally expressed gene 3 was found as a tumor suppressor associated with the pathogenesis and progression in human meningiomas (12). LncRNA SNHG15 promotes colon cancer cell invasion and metastasis through blocking degradation of transcription factor Slug (13). LncRNA PVT1 facilitates tumorigenesis and progression via regulation of miR-128-3p/gremlin 1 axis and bone morphogenetic protein

(BMP) signaling pathway in glioma (14). A growing body of evidence has indicated the importance of lncRNAs for the progression of CC. At present, the mechanisms of metastasis associated lung adenocarcinoma transcript 1 (MALAT1), HOTAIR and CDKN2B antisense RNA 1 (ANRIL) lncRNAs in cervical cancer have been characterized in detail (15-17). Guo *et al* (15) demonstrated that HOTAIR was upregulated in cervical cancer tissues compared with peritumoral tissues. Suppression of HOTAIR reduced autophagy and reversal of epithelial-mesenchymal transition through the inhibition of the Wnt signaling pathway, which consequently enhanced radiotherapy sensitivity in CC. MALAT1 has been reported to be generally upregulated in CC and reduces the efficiency of radiation treatment on CC cell lines by interacting with miR-143 *in vitro* (16). LncRNA ANRIL was significantly increased both in CC tissues and cell lines and regulated CC cell proliferation, migration and invasion through the PI3K/Akt pathway (17).

The aforementioned studies demonstrated the impacts of lncRNAs on CC progression and their potential as treatment targets. lncRNA-AK001903 has been verified as an upregulated gene in active ulcerative colitis tissues by non-coding (NC) RNA microarray (18). However, the expression and function of lncRNA-AK001903 in CC and its correlation with prognosis of patients with CC remains to be elucidated. Hence, the present study aimed to investigate the roles of lncRNA-AK001903 in the progression of cervical cancer, which may provide a new target for the treatment of CC.

Materials and methods

Cell lines and tissue samples. Cervical cancer cell lines: Ca Ski, Hela, Siha, and C33a were purchased from Procell Life Science & Technology Co., Ltd. and the normal cell line H8 was purchased from Shanghai Yu Bo Biotech Co., Ltd. Ca Ski and H8 were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Gibco; Thermo Fisher Scientific Inc.). Hela, Siha and C33a were cultured in Minimum Essential Medium (Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% heat-inactivated FBS. Penicillin (100 U/ml)/streptomycin (100 μg/ml) (GE Healthcare Life Sciences) was used in the culture medium and cells were grown in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells were passed to the next generation every three days. In the present study, tissues were collected from Department of Gynecological Oncology in Sun Yat-sen Memorial Hospital (Guangzhou, China) between June 2016 and May 2017. The inclusion criteria of the patients were as follows: i) Females diagnosed with CC using the International Federation of Gynecology and Obstetrics (FIGO, 2018) system (19); and ii) underwent biopsy or trachelectomy surgery. The exclusion criteria of the patients were as follows: i) Diagnosed with other tumors; ii) underwent radiotherapy or chemotherapy or any other treatment prior to surgery; and iii) CC recurrence. Peritumoral tissue samples were taken at least 1 cm distal to tumor margins. Tissue histology was independently evaluated by two pathologists. A total of 29 CC tissues (mean age, 37.6 years; age range, 21-77 years) and peritumoral tissues were collected and immediately placed and stored in liquid nitrogen. All of the 29 CC tissues and peritumoral tissues were used for reverse-transcription quantitative (RT-q) PCR analysis, 3 CC tissues and corresponding peritumoral tissues were used for microarray analysis and 26 CC tissues were used for ISHH. All patients underwent biopsy or trachelectomy surgery at the Sun Yat-sen Memorial Hospital (Guangzhou, China). All specimens were obtained with the approval of the Sun Yat-sen Memorial Hospital Review Board (approval no. 2015132) (Guangzhou, China) and a written informed content was obtained from each patient.

RNA microarray. Human 12x135k Long Non-coding RNA Array was manufactured by NimbleGen Systems Inc. Each array represented all long transcripts, both protein coding mRNAs and lncRNAs in the human genome. In total, >23,000 lncRNAs were collected from the authoritative data sources including NCBI RefSeq (GRCh37 (hg19); https://www.ncbi.nlm.nih.gov/refseq/), UCSC (GRCh37 (hg19); http://genome.ucsc.edu/cgi-bin/hgTables), RNAdb 2.0 (http://research.imb.uq.edu.au/rnadb/), lncRNAs from literatures (20,21) and UCRs (NCBI Build 35 (hg17); http://users.soe.ucsc.edu/~jill/ultra.html).

RNA labeling and array hybridization. In total 3 CC tissues and 3 corresponding peritumoral tissues were used to synthesize double-stranded complementary DNA (cDNA). Double-strand cDNA (ds-cDNA) was synthesized from 5 μg of total RNA using a SuperScript ds-cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific Inc.) in the presence of 100 pmol oligo dT primers according to the manufacturer's instructions. Ds-cDNA was cleaned and labeled in accordance with the NimbleGen Gene Expression Analysis protocol (NimbleGen Systems, Inc.). Briefly, ds-cDNA was incubated with 4 μg RNase A at 37˚C for 10 min and cleaned using phenol:chloroform:isoamyl alcohol (25:24:1), followed by ice-cold absolute ethanol precipitation. The purified cDNA was quantified using NanoDrop ND-1000 (Thermo Fisher Scientific Inc.). For Cy3 labeling of cDNA, the NimbleGen One-Color DNA labeling kit (NimbleGen Systems, Inc.) was used according to the manufacturer's instructions detailed in the Gene Expression Analysis protocol (NimbleGen Systems, Inc.). In brief, 1 μg ds-cDNA was incubated for 10 min at 98˚C with 1 optical density (OD) of Cy3-9mer primer. Next, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs Inc.) were added and the mixture incubated at 37˚C for 2 h. The reaction was stopped by 0.5 mol/l EDTA (0.1 times the volume of the previous mixture), and the labeled ds-cDNA was purified by isopropanol/ethanol precipitation. Microarrays were hybridized at 42˚C for 16-20 h with 4 μg of Cy3 labelled ds-cDNA in hybridization buffer/hybridization component A (NimbleGen Systems, Inc.) in a hybridization chamber (hybridization system; NimbleGen Systems, Inc.).

Microarray wash, scanning and data extraction. Washing was performed three times using the SeqCap EZ Hybridization and Wash kit (cat. no. 05634261001; NimbleGen Systems, Inc.). After being washed in an ozone-free environment, the slides were scanned using the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC.). Raw data were extracted as pair files using NimbleScan software version 2.5 (Roche

Table I. siRNA sequences used in the present study.

siRNA set	Sequence	
	Sense (5'-3')	Antisense (5'-3')
siRNA-AK001903-713	GCCCACACCAAUCUUAGAATT	UUCUAAGAUUGGUGUGGGCTT
siRNA-AK001903-1306	CCACAUGUCUCAGCUAUAUTT	AUAUAGCUGAGACAUGUGGTT-
siRNA-AK001903-1051	CGGACCCAUAUUAUCAUAUTT-	AUAUGAUAAUAUGGGUCCGTT
siRNA-AK001903-1605	GCAGUUCUUUAACCAAUGUTT	ACAUUGGUUAAAGAACUGCTT
siRNA-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Si, small interfering; NC, negative control.

NimbleGen, Inc.) and normalized through quantile normalization and the Robust Multichip Average algorithm included in the NimbleScan software. All gene level files were imported into GeneSpring GX software version. 11.5.1 (Agilent Technologies Inc.) or further analysis. Differentially expressed lncRNAs with statistical significance between two groups were identified through volcano plot filtering. Differentially expressed lncRNAs between two samples were identified through fold change filtering. P-value was calculated using the paired *t*-test. The threshold set for up- and downregulated genes was a fold change ≥2.0 and a P-value ≤0.05. Hierarchical clustering was performed using the GeneSpring GX software version 11.5.1 (Agilent Technologies Inc.).

Transfection. A total of 4 AK001903-specific small interfering (si)RNAs (si-AK001903-713, siRNA-AK001903-1306, siRNA-AK001903-1051 and siRNA-AK001903-1605) were used to knock down lncRNA-AK001903, and a non-targeting siRNA (si-NC) oligonucleotide was used as a negative control (all Shanghai Gene Pharma Co. Ltd.). Sequences were listed in Table I. Ca Ski were plated at 60% confluence ($5x10^5$ cells/well) on a 6-well plate. The following day, the culture medium was replaced with 500 μl of Opti-MEM (Gibco; Thermo Fisher Scientific Inc.). A total of 250 μl of Opti-MEM and 5 μl of Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific Inc.) were mixed in one tube, 20 pM siRNA (Shanghai GenePharma Co. Ltd.) and 250 μl of Opti-MEM were mixed in the other tube. Then they were combined and incubated for 5 min at room temperature. After 6 h incubation in 37˚C, the transfection medium was replaced with 2 ml of standard growth medium. Cells were haversted 48 h post transfection and subsequent experiments performed.

RT-qPCR. Total RNA was extracted from tissues or cell lines (Ca Ski, Hela, Siha, C33a and H8) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed into cDNA using a PrimeScript® RT reagent kit with gDNA eraser (Takara Bio Inc.). The protocol for RT was as follows: 37˚C for 15 min, 85˚C for 5 sec and termination at 4˚C. Quantitative PCR was performed using an ABI 7500 Real-Time PCR system and a SYBR® Premix Ex TaqTM II kit (Takara Bio Inc.). PCR thermocycling conditions were as follows: predenaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 60˚C for 30 sec. β-actin was used as an endogenous reference. Fold-changes of the relative expression of target genes were calculated using $2^{-\Delta\Delta Cq}$ method (22). All experiments were performed in duplicate and repeated twice. Primers for quantitative PCR were presented in Table II.

ISHH. Tissues were fixed with 4% paraformaldehyde (with 0.1% Diethyl pyrocarbonate) at room temperature for 24 h. Then they were paraffin-embedded and sliced into 20-μm thick slices and spread over PLL-coated glass slides. The paraffin sections were deparaffinized and rehydrated and treated with 0.2 mol/l HCl at 37˚C for 20 min, 3 μg/ml proteinase K (PCR grade; Roche Diagnostics) at 37˚C for 7 min followed by post-fixation with 4% paraformaldehyde (with 0.1% diethyl pyrocarbonate) for 5 min at room temperature. After acetylation in a solution consisting of 1.5% triethanolamine, 0.25% acetic anhydride, and 0.25% HCl at 40˚C for 4 h, the slides were washed by 5X SSC once for 15 min. Pre-hybridization was performed using the pre-hybridization solution (cat. no. AR0152; Boster Biological Technology Co. Ltd.) at 37˚C for 4 h. Subsequently, a digoxigenin-labeled oligonucleotide probe (5'-CCACAC CCACCTTCCCACATGCATGATA-3'; Shanghai GeneBio Co., Ltd.) was diluted to 200 nM using hybridization buffer (cat. no. AR0062; Boster Biological Technology Co. Ltd.) and hybridized with tissues for 16 h at 55˚C. Slides were washed twice at 55˚C for 30 min with a solution consisting of 50% formamide, 2X SSC and 0.01% Tween 20, and then 10 μg/ml RNase A was added at 37˚C for 1 h in buffer (0.5 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.01% Tween 20). Subsequently, the samples were washed in 2X SSC wash buffer with 0.01% Tween 20 at 55˚C for 30 min, followed by washing in 0.2X SSC wash buffer with 0.01% Tween 20 at 55˚C for 30 min. After additional washing in Tris-buffered saline (TBS, pH 7.6) and blocking in a buffer consisting of 10% Blocking Reagent (cat. no. 11096176001; Roche Diagnostics), 0.1 M maleate, 0.15 M NaCl, and 0.01% Tween 20 in TBS, DIG- labeled probes were detected by biotinylated digoxin antibody (cat. no. AR0147; Boster Biological Technology Co. Ltd.) at 37˚C for 1 h, streptavidin-biotin complex-peroxisome (SABC-POD; cat. no. AR0148; Boster Biological Technology Co. Ltd.) at 37˚C for 20 min and biotin-horseradish peroxidase (HRP) (cat. no. AR0149; Boster Biological Technology Co. Ltd.) at 37˚C for 20 min. The samples were stained using DAB

Table II. Reverse-transcription quantitative PCR primers.

Primer Set	Sequence/Assay ID
AK001903.1	Forward: 5'-AATCTGCCCACACCAATCTT-3'
	Reverse: 5'-CAGTGTGCTGAAATTCACCTG-3'
AI184890	Forward: 5'-GTCTCACTCTGTTGCCTGGG-3'
	Reverse: 5'-TGGGGACATTTGCGGAAATTTAT-3'
AK097842	Forward: 5'-AGGGTCTACATCGGCTCCTT-3'
	Reverse: 5'-CGTTCATGGTGCCGTCAAAG-3'
BG419628	Forward: 5'-GCACTGTGACCTCCCTGATC-3'
	Reverse: 5'-TGGGTCCACTTCGCAAATGA-3'
ASLNC01516	Forward: 5'-CGGACTGTTCTCCTTCCCAC-3'
	Reverse: 5'-GGGATTGCAGGTGTGATCCA-3'
ASLNC16271	Forward: 5'-AGCCTCCCTGTACAAGCAAC-3'
	Reverse: 5'-GTAAGTTCCCCGCCCTGTAG-3'
ASLNC14492	Forward: 5'-GGTAACGAATGCCCCTCCAA-3'
	Reverse: 5'-AGTAGGGCTGACTCTCCCAG-3'
ASLNC03532	Forward: 5'-CCTCCTCTCACCCAGGATCA-3'
	Reverse: 5'-GTGTTCCTGAGAAGGCCCTC-3'
BF675100	Forward: 5'-CAATGCTCAAACCACAGGCC-3'
	Reverse: 5'-TGTCATTTTCTCCTCGCCCC-3'
ASLNC17636	Forward: 5'-TGAGCCGAGATTGTGCCATT-3'
	Reverse: 5'-AAATGAGGCAGGTGACAGGG-3'
β-actin	Forward: 5'- TGGCACCCAGCACAATGAA-3'
	Reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

(cat. no. ZLI-9019; Origene Technologies Inc.) and images were captured using light microscopy (Nikon Corporation).

CCK-8 assay. Cell proliferation was assessed using the CCK-8 assay. Ca Ski were plated at $1x10^3$ cells/well on 96-well plates with three wells for each group. Cell viability was measured over 5 days using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies Inc.). A total of 10 μl of CCK-8 (5 mg/ml) was added to each well. After incubating for 4 h at 37˚C, the absorbance was determined at 450 nm.

Transwell assays. Cell migation and invasion were examined using Polycarbonate Membrane transwell inserts (Costar; Corning Inc.). The upper compartment was pre-coated with RPMI-1640 medium without serum for migation assay and Matrigel for 2 h in 37˚C (Corning Inc.) for invasion assay. After 48 h of transfection, $5x10^4$ Ca Ski cells were placed into the upper compartment. RPMI-1640 medium with 20% FBS was used in the lower compartment. Cells were incubated at 37˚C for 24 h for the migation assay and 48 h for the invasion assay. Then the compartment were fixed at room temperature with 4% paraformaldehyde for 20 min and stained with crystal violet (1 mg/ml) for 20 min at room temperature, and cells not crossing the membrane were cleaned. Images were captured using light microscopy (Nikon Corporationm).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.) and SPSS 20.0 software (IBM Corp.). All experiments were performed in triplicate. Comparisons between two groups were performed by paired sample t-tests. Three or more experimental groups were compared by one-way ANOVA followed by the post hoc Bonferroni test. The median value [tissues' CT (cycle threshold) value-corresponding β-actin CT value=7.2] of lncRNA-AK001903 expression level was used to divide patients into high and low lncRNA-AK001903 expression level groups. χ^2 test was used to compare the association between different clinical features and gene expression level. All data are presented as mean ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference.

Results

LncRNAs expression profile in CC. Hierarchical clustering demonstrated systematic variations in the expression of lncRNAs between CC tissues and corresponding peritumoral tissues (Fig. 1A). Volcano Plots were used to visualize the relationship between fold-change (magnitude of change) and statistical significance (which took both magnitude of change and variability into consideration) (Fig. 1B). According to the microarray expression profiling data, 453 distinctively dysregulated lncRNAs were detected in CC tissues compared to the controls (fold change, ≥2.0 and $P<0.05$) (Fig. 1A and B). The top 10 of 324 significantly upregulated and 129 significantly downregulated lncRNA transcripts are summarized in Table III. To validate the microarray analysis findings, 5 upregulated and 6 downregulated lncRNAs expression were detected

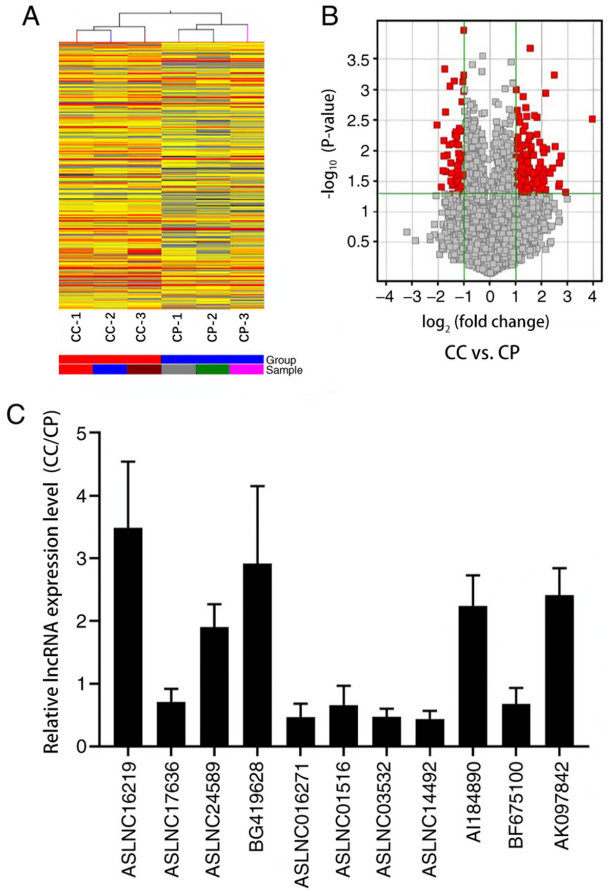

Figure 1. Differential expression of lncRNAs in CC tissues. (A) Hierarchical clustering analysis of lncRNAs that were differentially expressed between CC and paired peritumoral tissues (2.0-fold; P<0.05). Expression values are represented in shades with a gradient moving from red (high level) through yellow (median level) and finally on to green (low level), indicating expression above and below the median expression value across all samples (log scale 2, from-0.06 to +0.06), respectively. (B) Volcano Plot was performed to identify differentially expressed lncRNAs with statistical significance filtering between CC tissues and corresponding nontumor tissues. The threshold was fold change ≥2.0, P<0.05. The red point in the plot represents the differentially expressed lncRNAs with statistical significance. The gray point represents lncRNAs that do not satisfy this criterion. (C) A total of 11 differentially expression lncRNAs in 9 paired CC and peritumoral samples were detected by reverse-transcription quantitative PCR. β-actin was an internal control. The paired nontumor samples served as the negative control. Data are shown as mean ± SD. CC, cervical cancer; lncRNAs, long non-coding RNAs; CP, peritumoral tissues.

and compared between 9 pairs of CC tissues and their corresponding peritumoral tissues using RT-qPCR. These data confirmed that ASLNC16219 (seqname, AK001903), ASLNC24589 (seqname, U88892), BG419628 (seqname, chr20:1594825-1615675), AI184890 (seqname, HMlincRNA1030), AK097842 (seqname, HMlincRNA810) were upregulated in CC tissues compared to peritumoral tissues, whereas the expression of ASLNC17636 (seqname, AK055280), ASLNC016271 (seqname, AK021467), ASLNC01516 (seqname, NR_024345), ASLNC03532 (seqname, uc001esn), ASLNC14492 (seqname, AY927461) and BF675100 (seqname, chr12:92933675-92975575) was decreased (all P<0.05; Fig. 1C). Thus, the results strongly revealed that expression changes of lncRNAs were involved in the development of cervical cancer.

LncRNA-AK001903 is upregulated in CC tissues and cells and related to FIGO stage in patients with CC. Among the 20 most significantly differentially expressed lncRNAs (DE lncRNAs) in the CC tissues vs. CP (peritumoral tissues), the most notably upregulated one was lncRNA-AK001903 (fold change, 15.547; Table III). To verify the roles of lncRNA-AK001903 in CC cells, expression of lncRNA-AK001903 in CC cell lines and the normal cell line H8 were determined by RT-qPCR. The results demonstrated that Ca Ski exhibited significantly higher expression level of lncRNA-AK001903 compared with C33a, Hela and Siha cells. Besides, all these cervical cancer cell lines expressed higher level of lncRNA-AK001903 compared with H8 cells (Fig. 2A). In addition, lncRNA-AK001903 expression was assessed in 29 CC tissues and their corresponding peritumoral tissues to confirm the expression change of lncRNA-AK001903 in CC tissues. The result revealed significantly higher levels of lncRNA-AK001903 gene expression compared with corresponding peritumoral tissues (Fig. 2B). Clinicopathologic features of the 29 patients with CC were shown in Table IV. However, according to the results of RT-qPCR, the high and low expression of AK001903 in tumor tissues appeared to be independent of the patient's age, tumor size, FIGO stage, lymph node metastasis, differentiation, and whether the tumor was confined to the cervix (Table IV). These results suggested the probable oncogenic role of lncRNA-AK001903 in cervical cancer tumorigenesis. In addition, an ISHH probe of lncRNA-AK001903 was designed and synthesized to examine the expression differences among CC tissues. The results demonstrated that lncRNA-AK001903 was mostly located in the nucleus of cells and that high lncRNA-AK001903 expression was associated with advanced FIGO stage (Fig. 2C).

LncRNA-AK001903 regulates the cell proliferation, migration, and invasion in Ca Ski cells. As lncRNA-AK001903 was highly expressed in CC cells and tissues compared with H8 cells and peritumoral tissues and related to the FIGO stage of CC, further experimentation was performed to investigate whether lncRNA AK001903 may be a potential oncogene during the progression of CC. Four siRNAs targeting lncRNA-AK001903 at different sites (Table I) were designed and transfected into Ca Ski cells due to the highest expression of lncRNA-AK001903 in Ca Ski. The results demonstrated that of the 4 siRNAs used siRNA-lncRNA-AK001903-1605 produced the most effective interference of lncRNA-AK001903 expression and was then used for further experimentation (Fig. 3A). Next, the effects of knockdown of lncRNA-AK001903 on the proliferation, invasion and migration of Ca Ski cells was investigated. The CCK-8 assay demonstrated that lncRNA-AK001903 knockdown significantly inhibited the proliferation of lncRNA-AK001903 compared to the si-NC group (Fig. 3B). Transwell assay results demonstrated that the ability of migration and invasion were suppressed by knockdown of lncRNA-AK001903 (Fig. 3C).

Discussion

Cancer is a complex disease, involving various changes in gene expression (23,24). These gene expression changes cause cancer development, including metastasis (25), cell proliferation (26), invasion (27), and angiogenesis (28).

Table III. Top 10 significantly up- and downregulated lncRNAs in CC[a] vs. CP[b].

Seq name	P-value	Fold change[c]	MOR[d]	CC	CP	Associated	Source	Relationship
AK001903	0.003	15.547	Up	1,570.119	105.897		misc_lncRNA	Intergenic
HMlincRNA1030	0.046	7.559	Up	1,336.257	151.717		lincRNA	Intergenic
U88892	0.012	6.743	Up	657.814	97.620		misc_lncRNA	Intron sense-overlapping
HMlincRNA810	0.015	6.467	Up	796.874	136.080		lincRNA	Exon sense-overlapping
chr20:1594825-1615675	0.033	6.414	Up	1,263.708	220.417		lincRNA	Intron sense-overlapping
HMlincRNA1030	0.037	6.212	Up	1,382.065	221.246		lincRNA	Intergenic
uc010ldj	0.019	5.369	Up	2,010.306	385.026	HSP27[e]	UCSC_knowngene	Exon sense-overlapping
uc010dld	0.023	5.025	Up	397.059	81.809	TUBB6[f]	UCSC_knowngene	Exon sense-overlapping
NR_024204	0.009	4.565	Up	3,247.554	680.789	NCRNA00152[g]	RefSeq_NR	Intergenic
DQ786233	0.043	4.469	Up	603.588	113.588		misc_lncRNA	Intergenic
NR_024345	0.004	4.153	Down	1,615.112	7,101.768	C12orf27[h]	RefSeq_NR	Intergenic
AK021467	0.039	3.649	Down	267.197	1,035.963		misc_lncRNA	Intron sense-overlapping
AY927461	0.021	3.490	Down	496.854	1,600.906		misc_lncRNA	Intron sense-overlapping
uc001esn	0.012	3.480	Down	93.238	318.969	AX746564	UCSC_knowngene	Intergenic
chr12:92933675-92975575	0.007	3.476	Down	109.867	383.899		lincRNA	Exon sense-overlapping
AK055280	0.000	3.327	Down	273.747	910.918		misc_lncRNA	Intron sense-overlapping
uc003wog	0.021	3.321	Down	236.854	840.568	LOC154822	UCSC_knowngene	Natural antisense
chr3:64987550-65012925	0.002	3.274	Down	81.832	274.405		lincRNA	Intergenic
LIT1571	0.012	3.110	Down	257.196	806.208		RNAdb	Intergenic
HMlincRNA1338	0.008	2.965	Down	115.321	347.049		lincRNA	Intergenic

[a]CC, cervical cancer tissues; [b]CP, cervical peritumoral tissues; [c]Fold change, absolute ratio (no log scale) of normalized intensities between two conditions; [d]MOR, mode of regulation; [e]HSP27, heat shock protein 27; [f]TUBB6, tubulin beta 6 class V; [g]NCRNA00152, cytoskeleton regulator RNA; [h]C12orf27, HNF1A antisense RNA 1.

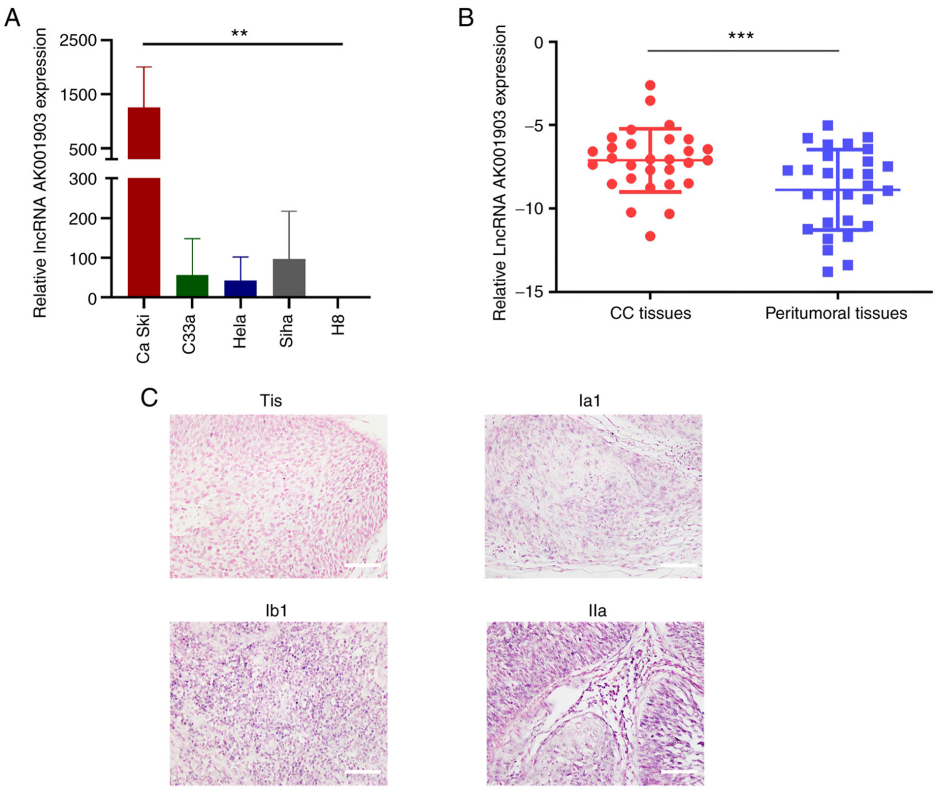

Figure 2. LncRNA-AK001903 was upregulated in CC cells and tissues and was related to FIGO stage. (A) LncRNA-AK001903 expression level was determined in CC Cells and H8 by reverse-transcription quantitative PCR. H8 cells served as the negative control. (B) LncRNA-AK001903 had a high expression in 29 CC tissues compared with their corresponding peritumoral tissues. β-actin was an internal control. (C) A total of 26 CC tissues were fixed and sectioned into slides then used for ISHH assay. The results demonstrated that high lncRNA-AK001903 expression was associated with advanced FIGO stage. Scale bar, 100 μm. Data are shown as the mean with SD. (**P<0.01; ***P<0.001). CC, cervical cancer; lncRNA, long non-coding RNA; ISH, *in situ* hybridization; Tis, tumor *in situ* [according to FIGO (2018)].

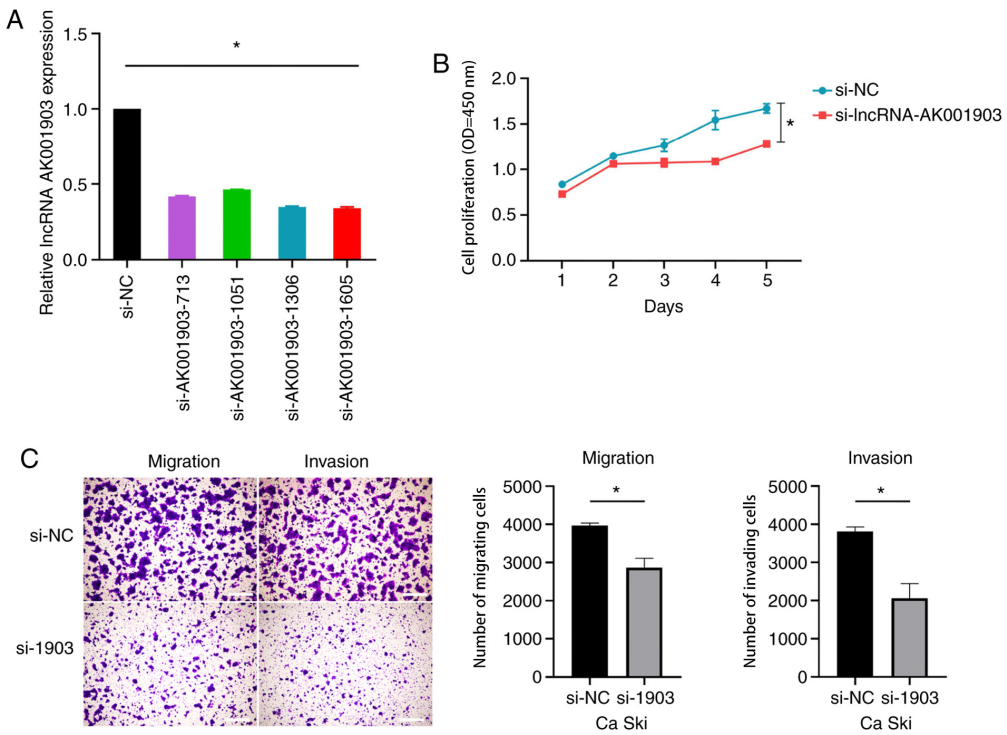

Figure 3. Effects of lncRNA-AK001903 silencing on CC progression. (A) Ca Ski cells were transfected with siRNA-lncRNA-AK001903-713, siRNA-lncRNA-AK001903-1306, siRNA-lncRNA-AK001903-1051, siRNA-lncRNA-AK001903-1605 or siRNA-NC. LncRNA-AK001903 expression was analyzed by reverse-transcription quantitative PCR at 48 h post-infection. β-actin was an internal control. (B and C) Ca Ski cells were transfected with siRNA-lncRNA-AK001903-1605 or siRNA-NC and then analyzed by the CCK-8 assay and transwell assay, respectively. Scale bar, 1,000 μm. Data are shown as mean ± SD. *P<0.05. CC, cervical cancer; lncRNA, long non-coding RNA; si, small interfering; NC, negative control; OD, optical density.

Table IV. Clinicopathologic features of CC tissues and corresponding peritumoral tissues (n=29).

Features	Low expression	High expression	P-value
Age, years			0.139
>50	4	9	
≤50	10	6	
Tumor size, cm			0.893
≥3	9	10	
<3	5	5	
FIGO stage, R			0.264
≥II	4	8	
<II	10	7	
Lymphatic metastasis			0.924
Yes	3	3	
No	11	12	
Differentiation			0.700
Low	4	6	
Moderate or high	10	9	
Confined to the cervix			0.837
No	8	8	
Yes	6	7	

CC, cervical cancer.

Numerous large-scale discovery studies have demonstrated the prospect of using lncRNAs as diagnostic and prognostic biomarkers, even in successful development of RNAi-based and oligo-based drugs (29,30). For example, lncRNA highly up-regulated in liver cancer level in blood and tissues may be used to detect liver cancer (31,32). HOTAIR in tissues can used as a prognostic marker for overall survival in breast cancer (11). Treatment with MTL-CEBPA (a small activating RNA drug) in mice lowered hepatocellular carcinoma tumor burden and improved clinically relevant parameters of liver function (29). As for the lncRNAs in cervical cancer, increasing numbers of reports have verified that lncRNAs are identified as potential biomarkers for cancer prognosis, invasion, metastasis, chemo-resistance and radio-resistance (33). Functional analysis of lncRNAs associated with CC progression may provide in depth understanding on the progression of CC (34).

Genome-wide microarray analysis has been used to identify the differentially expressed genes with higher diagnostic ability in tissues and cells, which facilitates the exploration of molecular mechanisms of tumor development (35). In the present study, microarray analysis was used to screen the aberrant lncRNAs in 3 CC patients to distinguish them in cancer tissues from corresponding peritumoral tissues. The results demonstrated that 453 lncRNAs (324 upregulated and 129 downregulated lncRNAs) were differentially expressed between CC tissues and peritumoral tissues from the same patients. A total of 6 downregulated and 5 upregulated lncRNAs were further verified by RT-qPCR in the present study and the results in 9 pairs of CC and peritumoral tissues were consistent with the microarray analysis.

It was recently reported that abnormal expression of lncRNAs, such as HAND2-AS1 and DLX6-AS1 serve an important role in the occurrence and development of CC (36-38). The change of cell phenotype depends on the influence of gene expression regulation (10). In the present study the most significantly upregulated lncRNA lncRNA-AK001903 was used to perform further experiments. The findings of the present study revealed that lncRNA-AK001903 was significantly upregulated in CC cell lines compared to normal cell line H8 and CC tissues compared to peritumoral tissues by RT-qPCR, and the level of lncRNA-AK001903 in CC tissues by ISHH was associated with FIGO (2018) stage. The aforementioned findings of the present study indicated that lncRNA-AK001903 may be a novel and effective biomarker in CC.

In the present study, the biological function of lncRNA-AK001903 in CC was explored. The CCK-8 assay demonstrated that knockdown of lncRNA AK001903 inhibited proliferation of Ca Ski cells, which indicated that lncRNA-AK001903 serves a crucial role in CC development. In the present study, transwell assays demonstrated that lncRNA-AK001903 promoted cell invasion and migration in CC. Considering Ca Ski is a cervical cancer cell line which was established from cells from a metastasis in the small bowel mesentery (39), lncRNA-AK001903 may act as an oncogene in the progression of CC and is a promising therapeutic target for the treatment of patients with CC.

In addition, the origin of lncRNA-AK001903 indicated that it was a long intergenic noncoding RNA (lincRNA) (20,40). LincRNAs, transcribed from intergenic regions of the genome, are the most abundant class of lncRNAs found in over 10,000 species so far (41). The potential mechanisms of lincRNA function include co-transcriptional regulation, regulation of gene expression in *cis* or in *trans* through recruitment of proteins or molecular complexes, titration of RNA-binding factors, and activation of posttranscriptional regulation by pairing with other RNAs (42). Atianand *et al* (43) discovered that erythroid prosurvival; also known as Ttc39aos1 was associated with chromatin at regulatory regions of immune response genes to control nucleosome positioning and repress transcription. TNFα and hnRNPL related immunoregulatory LincRNA exerted important roles in the innate immune response and inflammatory diseases in humans through combining with heterogenous nuclear ribonucleoprotein L to form a ribonucleoprotein complex to regulate tumor necrosis factor α induction (44). However, the mechanism of how lncRNA-AK001903 promotes cell proliferation, invasion and migration and possibly signal pathways remains unclear. Recently, increasing studies have reported lncRNAs were able to act as competing endogenous RNAs (ceRNAs) to competitively binding with microRNAs (miRNAs) to relieve the translation repression of targeted mRNAs induced by the common miRNAs and their downstream pathways (45-47). Nevertheless, whether lncRNA-AK001903 also regulates the progression of cervical cancer by acting as a ceRNA remains to be further explored.

The clinical application of lncRNA-AK001903 needs to be further explored due to the limitation of the present study of

not tracking the prognostic differences among patients with different expression levels.

In summary, the present study demonstrated that lncRNA AK001903 is a potential oncogene in CC. LncRNA AK001903 was able to promote CC tumor proliferation, migration and invasion and may act as therapeutic target and auxiliary criteria for evaluating FIGO stage.

Acknowledgments

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 81572575), Guangdong province Natural Scientific Grant (grant no. 2016A020215059), Special Support for Guangdong College Students' innovation and entrepreneurship training program (grant no. 1055813194), National College Students' innovation and entrepreneurship training program (grant no. 201310558097) and Guangdong clinical teaching base teaching program (grant no. 2018JD004).

Availability of data and materials

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

Authors' contributions

TY, XF and GZ contributed to the conception of the study and designed the experiments. YW, QX, XF and GZ performed the experiments. GZ, XF and RL contributed significantly to analysis and manuscript preparation. ZL and YW made substantial contributions to the acquisition of patient tissues and patient data. ZL analyzed and interpreted the patient data. GZ, TY and ZL revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All specimens were obtained with the approval of the Sun Yat-sen Memorial Hospital Review Board (approval number, 2015132) (Guangzhou, China). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ and Muñoz N: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12-19, 1999.
3. Moon JY, Song IC, Ko YB and Lee HJ: The combination of cisplatin and topotecan as a second-line treatment for patients with advanced/recurrent uterine cervix cancer. Medicine (Baltimore) 97: e0340, 2018.
4. Jabbar B, Rafique S, Salo-Ahen OMH, Ali A, Munir M, Idrees M, Mirza MU, Vanmeert M, Shah SZ, Jabbar I and Rana MA: Antigenic peptide prediction from E6 and E7 oncoproteins of HPV types 16 and 18 for therapeutic vaccine design using immunoinformatics and MD simulation analysis. Front Immunol 9: 3000, 2018.
5. US Preventive Services Task Force; Curry SJ, Krist AH, Owens DK, Barry MJ, Caughey AB, Davidson KW, Doubeni CA, Epling JW Jr, Kemper AR, *et al*: Screening for Cervical Cancer: US Preventive Services Task Force Recommendation Statement. JAMA 320: 674-686, 2018.
6. Zheng R, Zeng H, Zhang S, Chen T and Chen W: National estimates of cancer prevalence in China, 2011. Cancer Lett 370: 33-38, 2016.
7. Di Gesualdo F, Capaccioli S and Lulli M: A pathophysiological view of the long non-coding RNA world. Oncotarget 5: 10976-10996, 2014.
8. Sun L, Luo H, Liao Q, Bu D, Zhao G, Liu C, Liu Y and Zhao Y: Systematic study of human long intergenic non-coding RNAs and their impact on cancer. Sci China Life Sci 56: 324-334, 2013.
9. Ponting CP, Oliver PL and Reik W: Evolution and functions of long noncoding RNAs. Cell 136: 629-641, 2009.
10. Liu H, Luo J, Luan S, He C and Li Z: Long non-coding RNAs involved in cancer metabolic reprogramming. Cell Mol Life Sci 76: 495-504, 2019.
11. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, *et al*: Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464: 1071-1076, 2010.
12. Zhang X, Gejman R, Mahta A, Zhong Y, Rice KA, Zhou Y, Cheunsuchon P, Louis DN and Klibanski A: Maternally expressed gene 3, an imprinted noncoding RNA gene, is associated with meningioma pathogenesis and progression. Cancer Res 70: 2350-2358, 2010.
13. Jiang H, Li T, Qu Y, Wang X, Li B, Song J, Sun X, Tang Y, Wan J, Yu Y, *et al*: Long non-coding RNA SNHG15 interacts with and stabilizes transcription factor Slug and promotes colon cancer progression. Cancer Lett 425: 78-87, 2018.
14. Fu C, Li D, Zhang X, Liu N, Chi G and Jin X: LncRNA PVT1 facilitates tumorigenesis and progression of glioma via Regulation of MiR-128-3p/GREM1 axis and BMP signaling pathway. Neurotherapeutics 15: 1139-1157, 2018.
15. Guo X, Xiao H, Guo S, Li J, Wang Y, Chen J and Lou G: Long noncoding RNA HOTAIR knockdown inhibits autophagy and epithelial-mesenchymal transition through the Wnt signaling pathway in radioresistant human cervical cancer HeLa cells. J Cell Physiol 234: 3478-3489, 2019.
16. Zhu P, Wang FQ and Li QR: Correlation study between long non-coding RNA MALAT1 and radiotherapy efficiency on cervical carcinoma and generation of radiotherapy resistant model of cancer. Eur Rev Med Pharmacol Sci 22: 5140-5148, 2018.
17. Zhang D, Sun G, Zhang H, Tian J and Li Y: Long non-coding RNA ANRIL indicates a poor prognosis of cervical cancer and promotes carcinogenesis via PI3K/Akt pathways. Biomed Pharmacother 85: 511-516, 2017.
18. Wu F, Huang Y, Dong F and Kwon JH: Ulcerative colitis-associated long noncoding RNA, BC012900, regulates intestinal epithelial cell apoptosis. Inflamm Bowel Dis 22: 782-795, 2016.
19. Bhatla N, Aoki D, Sharma DN and Sankaranarayanan R: Cancer of the cervix uteri. Int J Gynaecol Obstet 143 (Suppl 2): S22-S36, 2018.
20. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, *et al*: Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458: 223-227, 2009.
21. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, *et al*: Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 106: 11667-11672, 2009.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

23. Huarte M: The emerging role of lncRNAs in cancer. Nat Med 21: 1253-1261, 2015.
24. Esquela-Kerscher A and Slack FJ: Oncomirs-microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
25. Wang H, Huo X, Yang XR, He J, Cheng L, Wang N, Deng X, Jin H, Wang N, Wang C, *et al*: STAT3-mediated upregulation of lncRNA HOXD-AS1 as a ceRNA facilitates liver cancer metastasis by regulating SOX4. Mol Cancer 16: 136, 2017.
26. Zhuang C, Ma Q, Zhuang C, Ye J, Zhang F and Gui Y: LncRNA GClnc1 promotes proliferation and invasion of bladder cancer through activation of MYC. FASEB J 33: 11045-11059, 2019.
27. Zhang J, Liu H, Hou L, Wang G, Zhang R, Huang Y, Chen X and Zhu J: Circular RNA_LARP4 inhibits cell proliferation and invasion of gastric cancer by sponging miR-424-5p and regulating LATS1 expression. Mol Cancer 16: 151, 2017.
28. Xu Y, Leng K, Yao Y, Kang P, Liao G, Han Y, Shi G, Ji D, Huang P, Zheng W, *et al*: A novel circular RNA, circ-CCAC1, contributes to CCA progression, induces angiogenesis, and disrupts vascular endothelial barriers. Hepatology: Aug 4, 2020 (Epub ahead of print).
29. Setten RL, Rossi JJ and Han SP: The current state and future directions of RNAi-based therapeutics. Nat Rev Drug Discov 18: 421-46, 2019.
30. Lavorgna G, Vago R, Sarmini M, Montorsi F, Salonia A and Bellone M: Long non-coding RNAs as novel therapeutic targets in cancer. Pharmacol Res 110: 131-138, 2016.
31. Panzitt K, Tschernatsch MM, Guelly C, Moustafa T, Stradner M, Strohmaier HM, Buck CR, Denk H, Schroeder R, Trauner M and Zatloukal K: Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA. Gastroenterology 132: 330-342, 2007.
32. Xie H, Ma H and Zhou D: Plasma HULC as a promising novel biomarker for the detection of hepatocellular carcinoma. Biomed Res Int 2013: 136106, 2013.
33. Dong J, Su M, Chang W, Zhang K, Wu S and Xu T: Long non-coding RNAs on the stage of cervical cancer (Review). Oncol Rep 38: 1923-1931, 2017.
34. Galvão M and Coimbra EC: Long noncoding RNAs (lncRNAs) in cervical carcinogenesis: New molecular targets, current prospects. Crit Rev Oncol Hematol 156: 103111, 2020.
35. Schmidt U and Begley CG: Cancer diagnosis and microarrays. Int J Biochem Cell Biol 35: 119-124, 2003.
36. Slack FJ and Chinnaiyan AM: The Role of Non-coding RNAs in Oncology. Cell 179: 1033-1055, 2019.
37. Tian Y, Wang YR and Jia SH: Knockdown of long noncoding RNA DLX6-AS1 inhibits cell proliferation and invasion of cervical cancer cells by downregulating FUS. Eur Rev Med Pharmacol Sci 23: 7307-7313, 2019.
38. Jin L, Ji J, Shi L, Jin S and Pei L: lncRNA HAND2-AS1 inhibits cancer cell proliferation, migration and invasion by downregulating ROCK1 in HPV-positive and negative cervical squamous cell carcinoma. Exp Ther Med 18: 2512-2518, 2019.
39. Pattillo RA, Hussa RO, Story MT, Ruckert AC, Shalaby MR and Mattingly RF: Tumor antigen and human chorionic gonadotropin in CaSki cells: A new epidermoid cervical cancer cell line. Science 196: 1456-1458, 1977.
40. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, *et al*: Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 106: 11667-11672, 2009.
41. Chen LL: Linking long noncoding RNA localization and function. Trends Biochem Sci 41: 761-772, 2016.
42. Ulitsky I and Bartel DP: lincRNAs: Genomics, evolution, and mechanisms. Cell 154: 26-46, 2013.
43. Atianand MK, Hu W, Satpathy AT, Shen Y, Ricci EP, Alvarez-Dominguez JR, Bhatta A, Schattgen SA, McGowan JD, Blin J, *et al*: A Long Noncoding RNA lincRNA-EPS acts as a transcriptional brake to restrain inflammation. Cell 165: 1672-1685, 2016.
44. Li Z, Chao TC, Chang KY, Lin N, Patil VS, Shimizu C, Head SR, Burns JC and Rana TM: The long noncoding RNA THRIL regulates TNFα expression through its interaction with hnRNPL. Proc Natl Acad Sci USA 111: 1002-1007, 2014.
45. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, García JA and Paz-Ares J: Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet 39: 1033-1037, 2007.
46. Song J, Ye A, Jiang E, Yin X, Chen Z, Bai G, Zhou Y and Liu J: Reconstruction and analysis of the aberrant lncRNA-miRNA-mRNA network based on competitive endogenous RNA in CESC. J Cell Biochem 119: 6665-6673, 2018.
47. Tay Y, Rinn J and Pandolfi PP: The multilayered complexity of ceRNA crosstalk and competition. Nature 505: 344-352, 2014.