Original Article
LINC01305 inhibits malignant progression of cervical cancer via miR-129-5p/Sox4 axis

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Abstract: Background: The association between LINC01305, a newly discovered long non-coding RNA (lncRNA), and cervical cancer (CC) has been poorly analyzed. In the present study, we revealed high expression of LINC01305 in CC by the cancer genome atlas (TCGA) and Gene Expression Omnibus (GEO), and dissected the related mechanisms. Methods: LINC01305, microRNA (miR) -129-5p and SRY-related high-mobility group box 4 (Sox4) mRNA levels were quantitated by quantitative reverse transcription-PCRy qRT-PCR). CC tissues and cell lines and corresponding controls were enrolled for the quantification of LINC01305 expression in CC. Effects of LINC01305 and miR-129-5p on cell proliferation, metastasis, and apoptosis were evaluated by MTT, colony formation, wound healing, Transwell and flow cytometry assays. Sox4 protein levels were tested by Western blot (WB). Bioinformatics analysis, RNA immunoprecipitation (RIP), RNA pull-down and dual-luciferase reporter (DLR) assay were performed to determine molecular mechanisms of LINC01305 in CC. Xenograft models of CC were constructed to evaluate the role of LINC01305 in vivo. Results: The expression of LINC01305 was evidently elevated in CC tissues and cell lines than that in controls and associated with clinicopathological features. Downregulating LINC01305 suppressed malignant phenotypes (proliferation, migration, invasion) of Hela and SiHa cells. In addition, silencing miR-129-5p by its inhibitor eliminated the inhibition of growth and metastasis induced by LINC01305 siRNA. Sox4 might serve as a direct target for miR-129-5p and was negatively regulated by miR-129-5p and LINC01305. Conclusion: LINC01305 acts as a competitive endogenous RNA (ceRNA) and regulates Sox4 via sponging miR-129-5p, contributing to the diagnosis and treatment of CC.

Keywords: lncRNA LINC01305, miR-129-5p, Sox4, cervical cancer

Introduction

Cervical cancer (CC) is a common gynaecological malignancy and a leading cause of cancer death among women worldwide [1, 2], with 500,000 newly diagnosed patients and 250,000 deaths per year. Human papillomavirus (HPV) vaccination is effective for preventing CC [3]; however, it has not been popularized in China, resulting in missed or delayed vaccinations of most women. The combination of surgical resection and chemotherapy is the preferred treatment to control prognosis of patients with CC, still, there is a risk of developing lymph node metastasis (LNM) and distant metastasis (DM), leading to poor survival and prognosis [4, 5]. Also, lacking effective screening biomarkers and techniques causes

poor diagnosis of CC [6]. Therefore, comprehending the molecular function and mechanism of CC and seeking potential therapeutic targets are of great significance.

Long-chain non-coding RNAs (lncRNAs), a new class of non-coding RNAs with a length of over 200 nt, play a role in chromatin modification [7, 8]. There is evidence that they are involved in many diseases, especially tumors [9, 10]. LncRNA JPX has been reported to regulate tumorigenesis and pulmonary metastases via miR-33A-5P/TWIST1 axis [11]. Moreover, lncRNA LINC00483 is involved in gastric cancer progression through regulating MAPK1 expression by sponging miR-490-3p [12]. LINC01305 is a newly discovered lncRNA located on human chromosome 2q31.1. It is highly expressed in

Table 1. Primer sequence

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
LINC01305	GCTCCTTCCGAAGTCTCGCT	ACTCCGCTAGCCAAGGACAC
miR-129-5p	GCGGCTTTTTGCGGTCTGG	GTGCAGGGTCCGAGGT
Sox4	CGAGTGGTTCTTCTGCGCTA	GGGCTGCTGGAAGGTAAACT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	ACACCATGGGGAAGGTGAAG	AAGGGGTCATTGATGGCAAC

CC and participates in epithelial-mesenchymal transition (EMT) by regulating PI3K/Akt signaling pathway [13]. Therefore, LINC01305 may be a potential target of CC.

The proposal of competitive endogenous RNA (ceRNA) theory has sped up lncRNA explorations [14]. LncRNAs regulate microRNAs (miRs) by competing for miR response elements (MREs) [15]. miR-129-5p is a reported tumor suppressor that is lowly expressed in CC [16]. We noticed that there were targeted binding sites between LINC01305 and miR-129-5p, suggesting that LINC01305 might be associated with CC via miR/mRNA axis.

Therefore, the potential mechanism of LINC0-1305 in CC was identified to inspire the development of new therapies.

Methods and data

Database analysis

Login to Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/gds/), and enter the keywords cervical cancer and lncRNA for initial screening. GSE63514 was selected for analysis. Series Matrix File(s) and GPL570 data were downloaded and merged into transcriptome data. Limma package was used to analyze differential lncRNAs. CC transcriptome data downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) were processed by edgeR package. Analyses in the two databases followed the standard of Fold Change = 1, P = 0.05.

Clinical data

Fifty-six patients with CC admitted to our hospital from January 2013 to January 2015 were enrolled. CC and adjacent tissues sampled during surgery were transported in liquid nitrogen and stored at -80°C. All patients signed the informed consent and had never received antitumor therapy. The present study was per-formed with the approval from the Medical Ethics Committee, and was in adherence to the Declaration of Helsinki [17].

Cell culture and transfection

Human cervical epithelial cell line (HcerEpic) and human CC cell lines (C33A, MS751, SiHa, HeLa and CaSki) were grown in RPMI-1640 medium (Gibco, NY, USA) comprising 10% fetal bovine serum (FBS, Invitrogen, NY, USA) at 37°C/5% CO_2. siLINC01305#1, siLINC01305#2, sil-INC01305#3 and negative control (si-NC), miR-129-5p mimics and NC-mimics, miR-129-5p inhibitor (miR-129-5p-inhibit) and NC-inhibit, LINC01305 overexpression plasmid (pcDNA-LINC01305) and negative control (Control) and LINC01305 inhibitor (sh-LINC01300), si-Sox4 and NC were all acquired from GenePharma, Shanghai, China. The cells were transfected with Lipofectamine 2000 (Invitrogen), and then harvested 24 hours later.

qRT-PCR

TRIzol reagent (Invitrogen, USA) was used to extract total RNA from samples. The purity, concentration and integrity were measured by an ultraviolet spectrophotometer (UV-2600) and agarose gel electrophoresis. Afterwards, total RNA was reverse transcribed with the Revertaid First Strand cDNA Synthesis kit. Part of cDNA obtained was stored and the other part was used for PCR amplification with SYBR-Green PCR Master Mix kit on an ABI 7500PCR. Three repeat wells were set up for each sample and the test was repeated three times. U6 and GAPDH served as internal references of miR and mRNA, respectively [18], and $2^{-\Delta\Delta ct}$ was used to analyze the data. Primer sequences were listed in **Table 1**.

CCK-8 assay

CCK-8 assay (Dojindo, Japan) was employed for determination of cell viability. Cells ($2 \times 10^3/100$ μL) were seeded into 96-well plates at the 0th, 24th, 48th and 72nd hour (37°C). Following the addition of CCK-8 reagent (10 μL), the cells in each well were cultured at 37°C for 2 hours. OD values at 450 nm were analyzed using a microplate reader (Bio-Rad, CA, USA).

Transwell assay

Transwell was performed to determine cell migration and invasion. The protocol is as follows: Transwell insert was used for invasion determination. After reaching ~80% confluence, cells were incubated with FBS-free medium in apical chamber coated with Martrigel (Corning, 1 mg/mL). Medium comprising 10% FBS (600 µL) was added into the basolateral chamber. After 24 hours, invaded and migrated cells were fixed with paraformaldehyde (4%) and dyed with crystal violet (0.5%), then counted under an Olympus microscope (40×10) under five random fields.

Fluorescence activated cell sorting (FACS)

Annexin-V-FITC apoptosis kit (BD, Franklin Lakes, USA) was utilized for apoptosis determination. Transfected cells digested with EDTA-free trypsin were resuspended in binding buffer (500 µL). Then, they were dyed with Annexin V/FITC and Propidium iodide (PI) (5 µL each) at room temperature for 15 min. The apoptosis was measured by FACS Calibur flow cytometer (BD Biosciences, CA, USA), and the test was repeated three times.

Western blot (WB)

Cells were lysed in RIPA buffer comprising 1% protease inhibitor. The proteins (20 µg) harvested were separated on a sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, USA). Afterwards, the membrane was sealed with 5% skim milk, followed by an overnight incubation with primary antibody in TBS at 4°C. Next, the membrane was incubated with secondary antibody at room temperature for another 1 hour. Finally, image visualization was performed with enhanced chemiluminescence reagent (ECL, Thermo, Rockford, USA).

Dual-luciferase reporter (DLR) assay

cDNA fragments comprising LINC01305-wild type (wt) or LINC01305-mutant (mut) were subcloned into the psi-CHECK2 luciferase reporter vector. miR-129-5p mimic or miR-129-5p inhibitor was co-transfected with LINC-01305 reporter vector (Invitogen, USA) into HEK293 cells. After 48 hours, firefly and renilla luciferase activities in cell lysates were measured with a DLR kit (Promega, USA). The activities of Sox4 and miR-129-5p were detected as described above.

RNA immunoprecipitation (RIP)

Using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA), RIP was conducted to identify the binding relationship between endogenous LINC01305 and miR-129-5p in HeLa cells. Whole cell lysates were immersed in RIP buffer comprising magnetic beads coupled with human anti-Ago2 antibody/control IgG. Proteins were digested with proteinase K, and RNA precipitates were resolved to purify RNA. At last, qRT-PCR was employed for quantification.

Statistical analysis

GraphPad 7 and SPSS20.0 were used for building figures and analyzing independent prognostic variables, respectively. Recorded as mean ± standard deviation (Mean ± SD), comparisons of continuous data were conducted by independent samples t test. Categorical data expressed by percentage (%) were analyzed by chi-square test (denoted by x^2). One-way analysis of variance (ANOVA) was adopted for multi-group comparison (denoted by F), Fisher's least significant difference-t (LSD-t) test for post-hoc pairwise comparison, repeated measures ANOVA for comparison among different time points (denoted by F), and Bonferroni for the post hoc test. Pearson test identified correlations between the genes, and overall survival was analyzed by K-M survival curve and Log-rank test. Significance was determined when probability (P) values were less than 0.05.

Results

High expression of LINC01305 in CC portends a poor prognosis

Differentially expressed lncRNAs in CC samples were screened from TCGA and GSE63514. A total of 1,034 lncRNAs were found in TCGA, 358 with high expression and 676 with low expression (**Figure 1A**). Whereas 11 lncRNAs were found in GSE63514, 5 with high expression and 6 with low expression (**Figure 1B**). Venn diagrams revealed that only one lncRNA
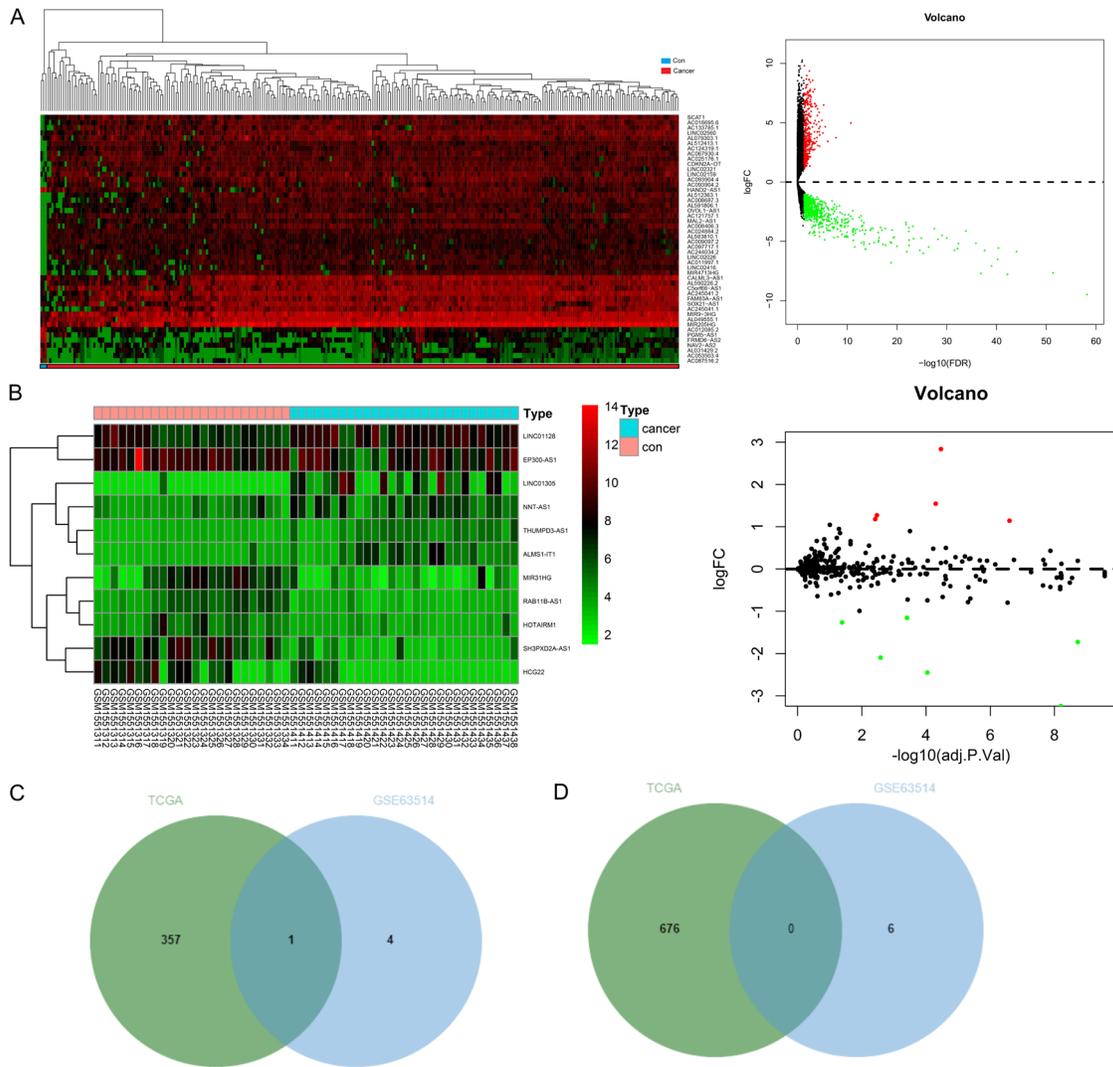
Figure 1. Screening of differentially expressed lncRNAs in CC from TCGA and GEO database. A. Differentially expressed lncRNAs in TCGA (only partially shown). B. Differentially expressed lncRNAs in GSE63514 of GEO. C. Venn diagram of highly expressed lncRNAs in TCGA and GSE63514. D. Venn diagram of lowly expressed lncRNAs in TCGA and GSE63514. CC: cervical cancer; TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus.

(LINC01305) showed high expression in both datasets, and there was no lowly expressed lncRNA available (**Figure 1C**, **1D**). Therefore, LINC01305 was used for further study. As shown in qRT-PCR, LINC01305 had a higher expression in CC tissues than that in controls, which was consistent with our findings from datasets (**Figure 2A**). Patients were allocated into high and low expression groups by median LINC01305 expression. Analyses of clinical data revealed that the highly expressed LINC-01305 was strongly associated with TNM staging and lymph node metastasis (LNM) (**Table 2**). Moreover, the 5-year survival in high expression group decreased evidently (**Figure 2B**). In

addition, a high expression of LINC01305 was revealed in CC cells by qRT-PCR, indicating that it may be an available biomarker of CC (**Figure 2C**).

Downregulating LINC01305 inhibits proliferation, invasion and migration, and enhances apoptosis

To determine whether LINC01305 was associated with CC, we established three si-LINC0-1305 vectors, and selected si-LINC01305#1 with the most significant inhibition to transfect SiHa and HeLa cells (**Figure 3A**, **3B**). Cell proliferation, invasion, migration and apoptosis were
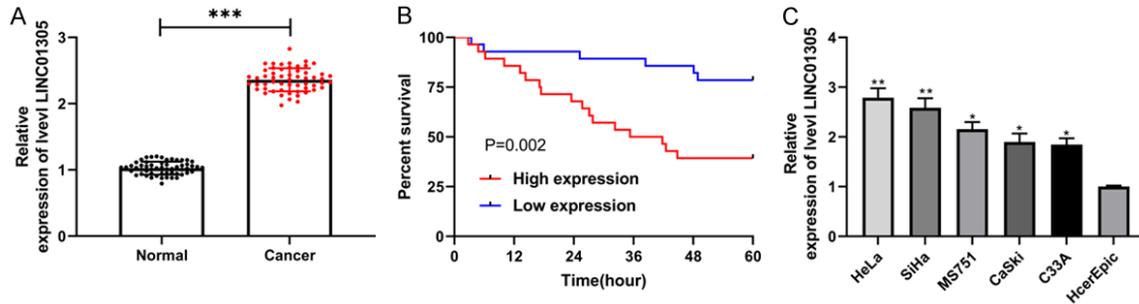
Figure 2. Association between LINC01305 expression and patient survival in CC. A. LINC01305 expression in CC tissues is quantified by qRT-PCR. B. Correlation between LINC01305 expression and patient survival in CC is identified by K-M survival curve. C. LINC01305 expression in CC cell lines is quantified by qRT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001. CC: cervical cancer; qRT-PCR: quantitative reverse transcription-PCR.

Table 2. Association between LINC01305 and pathological data

Factor	Relative expression of LINC01305		P
	High expression group (n = 28)	Low expression group (n = 28)	
Age (years)			0.265
≥ 60 (n = 20)	12	8	
< 60 (n = 36)	16	20	
Tumor size			0.397
≥ 5 cm (n = 19)	8	11	
< 5 cm (n = 37)	20	17	
Figo stage			0.003
I+II (n = 40)	15	25	
III+IV (n = 16)	13	3	
Differentiation			0.828
Poorly differentiated (n = 17)	10	7	
Moderately and highly differentiated (n = 39)	18	11	
Lymph node metastasis			0.002
Yes (n = 22)	16	5	
No (n = 34)	12	23	

tested. The proliferation of CC cells treated with si-LINC01305#1 was evidently inhibited, as shown by CCK-8 assay (**Figure 3C**, **3D**). In Transwell, the invasion and migration of transfected cells were remarkably suppressed (**Figure 3E**, **3F**). As shown in FACS, transfection of si-LINC01305#1 induced apoptosis (**Figure 3G**). These tests preliminarily reveal the inhibitory effect of downregulating LINC01305 on CC cells.

*LINC01305 functions as a miR-129-5p sponge*

lncRNAs regulate miRs by competing for MREs. We predicted potential miRs of LINC01305 through an online website to explore its potential mechanism in CC. The existence of targeted binding site between miR-129-5p and LINC-01305 was predicted by miRDB [19] and LncBase [20] (**Figure 4A**). Following RIP and DLR assays, it was found that both miR-129-5p and LINC01305 could be precipitated by Ago2 antibody (**Figure 4C**), and miR-129-5p-mimics decreased luciferase activity of LINC-01305 (**Figure 4B**, **4D**). miR-129 expression in si-LINC01305# treated cells was measured by qRT-PCR for validation (**Figure 4E**). These findings indicate that LINC01305 can function as a miR-129-5p sponge.

miR-129-5p can target Sox4

TargetScan [21], miRDB [19], miR-Walk [22], starBase [23], miRTarBase [24] were employed
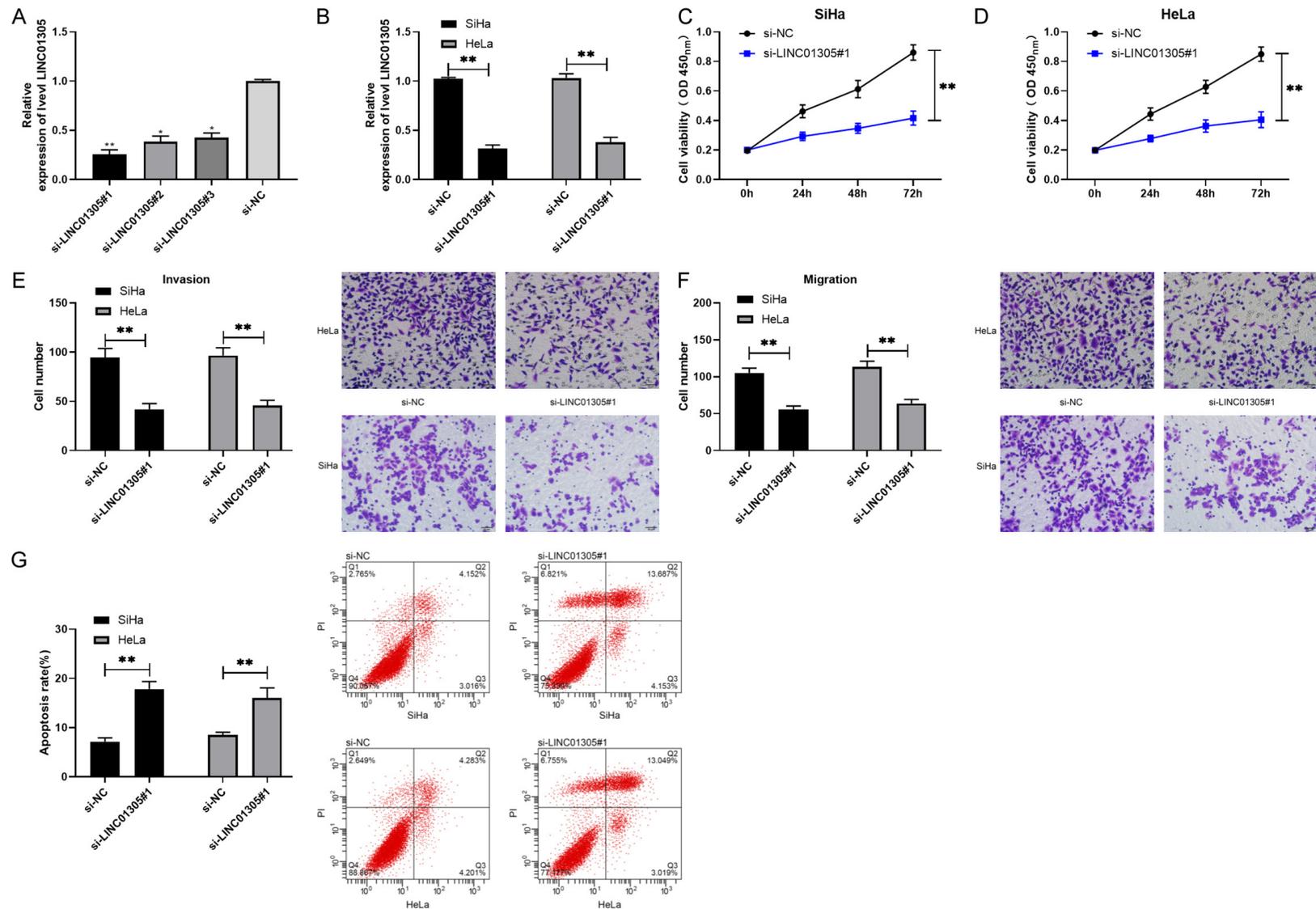
Figure 3. LINC01305 exhibits oncogenic function in CC. A. Relative expression of LINC01305 in si-LINC01305 vectors is quantified by qRT-PCR. B. Relative expression of LINC01305 in CC cells transfected with si-LINC01305#1 is quantified by qRT-PCR. C, D. Proliferation of cells transfected with si-LINC01305#1 is tested by CCK-8 assay. E, F. Invasion and migration of cells transfected with si-LINC01305#1 are tested by Transwell. G. Apoptosis of cells transfected with si-LINC01305#1 is tested by FACS. *P < 0.05, **P < 0.01. CC: cervical cancer; qRT-PCR: quantitative reverse transcription-PCR; FACS: fluorescence activated cell sorting.
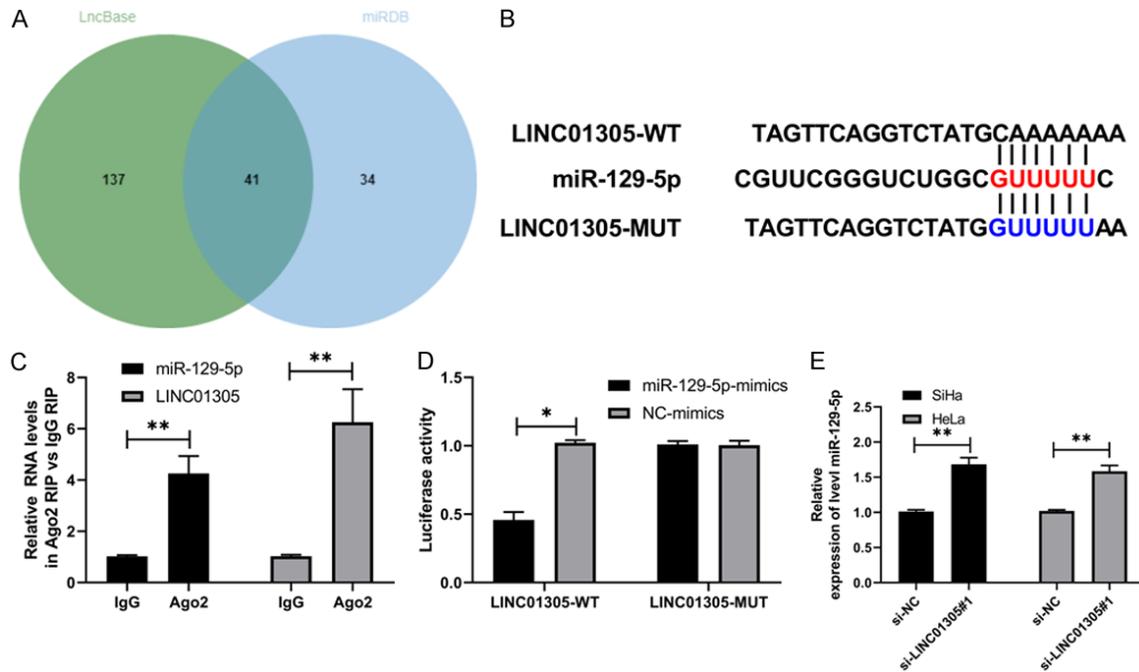
Figure 4. LINC01305 functions as a miR-129-5p sponge. A. Venn diagram of miRDB and LncBase predicting potential miRs of LINC01305. B. Potential binding sites between LINC01305 and miR-129-5p. C. Binding relationship between LINC01305 and miR-129-5p is confirmed by RIP. D. DLR assay reveals that LINC01305 binds to miR-129-5p in a targeted manner. E. miR-129-5p expression in CC cells transfected with si-LINC01305#1 is quantified by qRT-PCR. *P < 0.05, **P < 0.01. RIP: RNA immunoprecipitation; qRT-PCR: quantitative reverse transcription-PCR; CC: cervical cancer.

to screen downstream target genes of miR-129-5p, so as to explore the potential mechanism (**Figure 5A**). It turned out that there were target binding sites between Sox4 and miR-129-5p. Besides, DLR assay revealed a decrease in luciferase activity of Sox4-wt by miR-129-5p-mimics (**Figure 5B**, **5C**). Moreover, qRT-PCR and WB revealed that Sox4 decreased evidently in CC cells treated with miR-129-5p-mimics (**Figure 5D**, **5E**). Therefore, miR-129-5p is able to negatively regulate Sox4 expression in CC.

Up-regulation of LINC01305 induces CC via miR-129-5p/Sox4 axis

In view of the above findings, we speculated that LINC01305 might participate in CC via miR-129-5p/Sox4 axis. We conducted a rescue experiment to verify the existence of LINC01305/miR-129-5p/Sox4 axis. Malignant phenotypes of CC cells treated with miR-129-5p-mimics or si-Sox4 were evidently slowed down than those treated with pcDNA-NC, and apoptosis was enhanced. However, cells co-transfected with pcDNA-LINC01305 and miR-

129-5p-mimics or si-Sox4 presented no significant difference (**Figure 6A**, **6E**). WB demonstrated that Bax, Cle-caspase3 and Cle-caspase9 levels decreased and Bcl-2 levels increased by transfection of pcDNA-LINC01305; however, the co-transfection reversed these results (**Figure 7**). This indicates that LINC01305 participates in CC via the miR-129-5p/Sox4 axis.
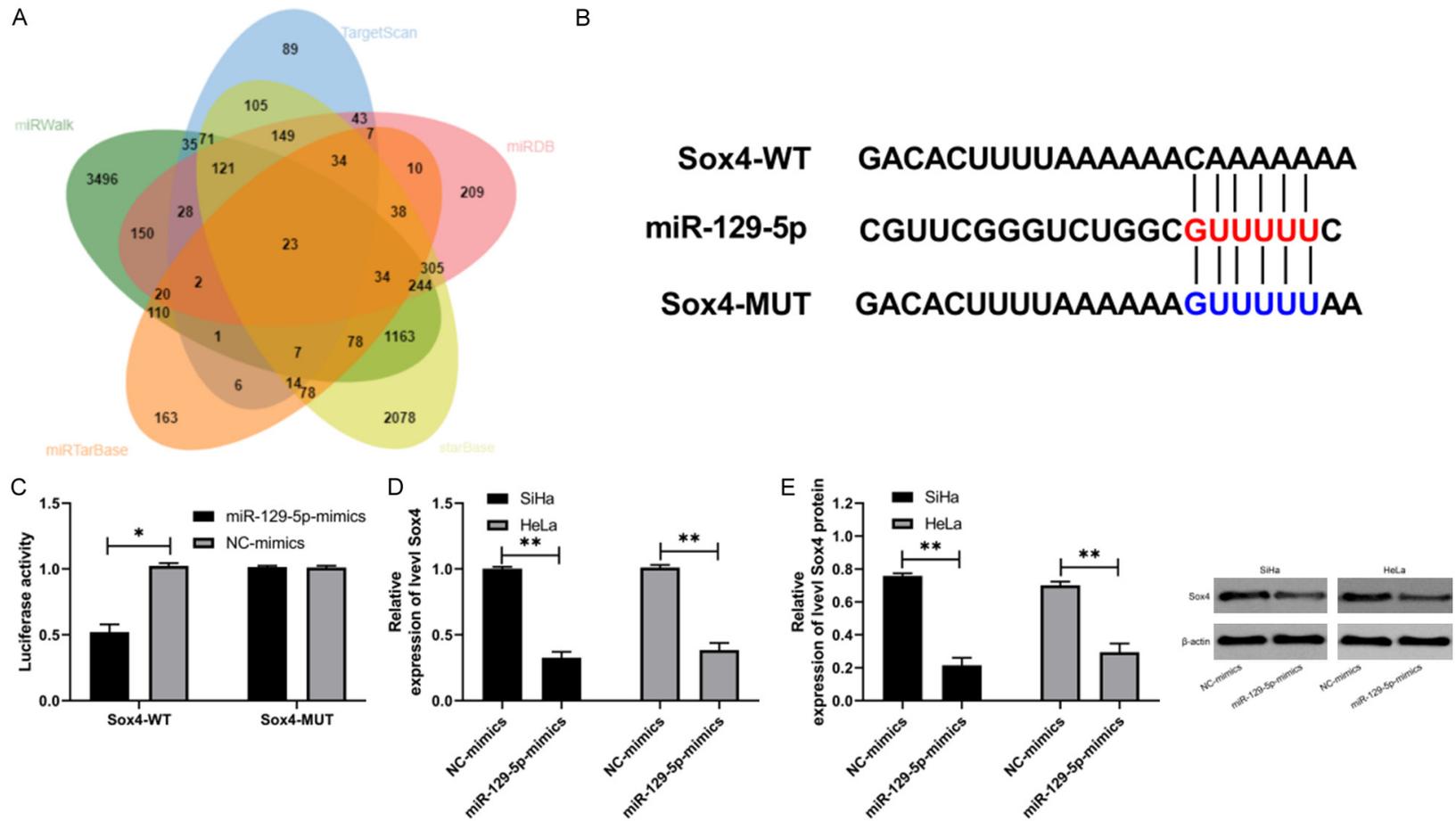
Correlations between LINC01305 and miR-129-5p and Sox4

miR-129-5p presented a low level in CC tissues, while Sox4 was the opposite. In addition, LINC01305 was reversely associated with miR-129-5p and positively associated with Sox4, and Sox4 and miR-129-5p were reversely associated (**Figure 8A-E**). This confirms the close relationship between LINC01305 and miR-129-5p/Sox4 axis.
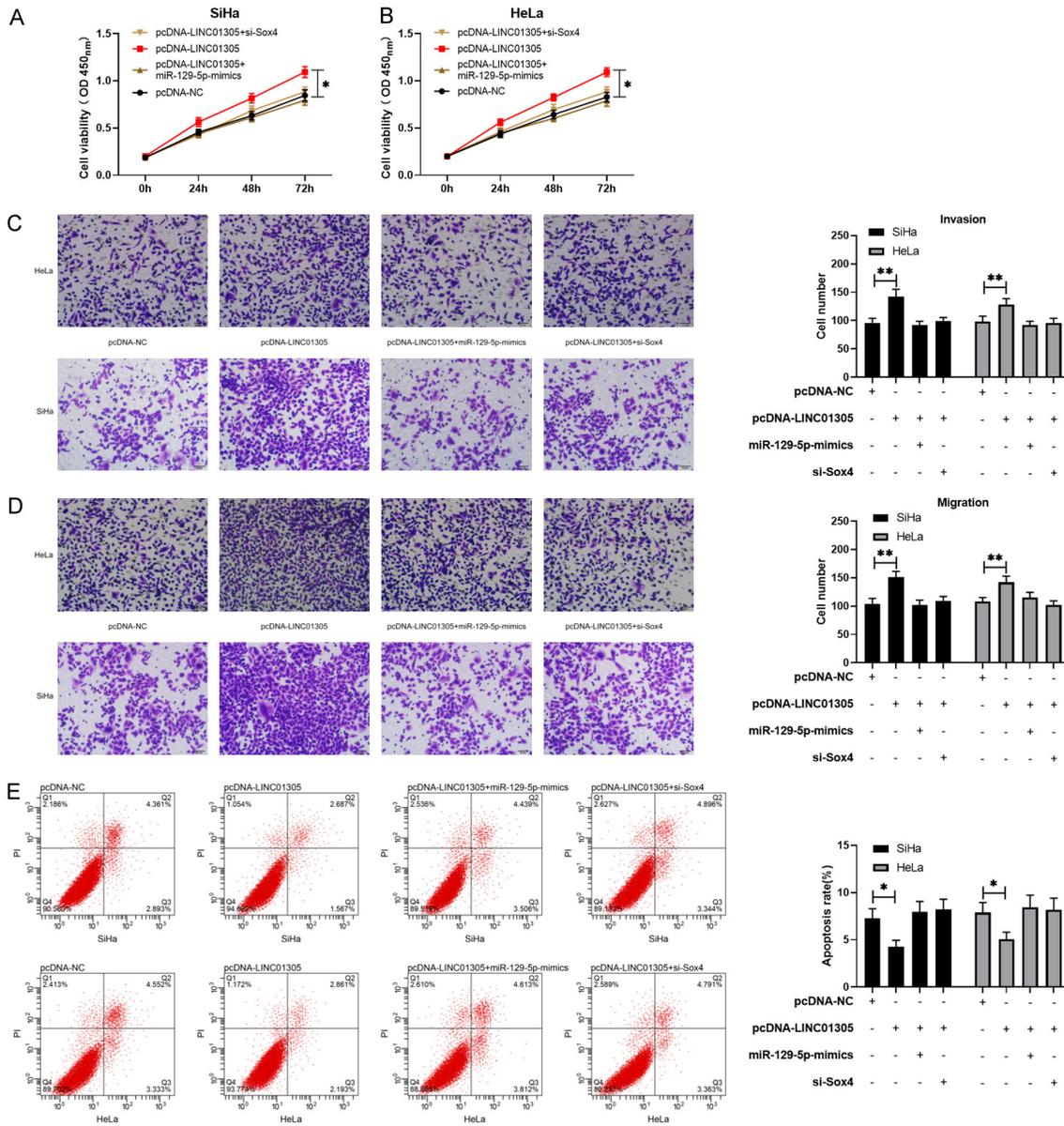
Discussion

Patients with metastatic CC, a highly malignant tumor among women, suffer from a poor prog-

Figure 5. miR-129-5p targets Sox4. A. Venn diagram of TargetScan, miRDB, miR-Walk, starBase, miRTarBase predicting potential target genes of miR-129-5p. B. Potential binding loci between miR-129-5p and Sox4. C. Binding relationship between LINC01305 and Sox4 is confirmed by RIP. D. Sox4 mRNA expression in cells transfected with miR-129-5p-mimics is quantified by qRT-PCR. E. Sox4 protein expression in cells transfected with miR-129-5p-mimics is quantified by WB. *P < 0.05, **P < 0.01. RIP: RNA immunoprecipitation; qRT-PCR: quantitative reverse transcription-PCR; WB: Western blot.

Figure 6. LINC01305 induces CC via miR-129-5p/Sox4 axis. A, B. Cell proliferation after co-transfection is tested by CCK-8 assay. C, D. Cell invasion and migration after co-transfection are tested by Transwell. E. Cell apoptosis after co-transfection is tested by FACS. *P < 0.05, **P < 0.01. CC: cervical cancer; FACS: fluorescence activated cell sorting.

nosis [25]. There is accumulating evidence that lncRNA is strongly related to CC and is a potential regulator of the disease [26, 27]. The present study proposed that LINC01305 presented a high expression and mediated CC via miR-129-5p/Sox4 axis, which is expected to be an available biomarker for its treatment.

LINC01305, a newly discovered lncRNA, has not been dissected. We noticed that the prognosis of patients with high expression of

LINC01305 was poor, which reminds us that LINC01305 may be a prognostic indicator of CC. Yan pointed out that LINC01305 is highly expressed in CC [13]. On this basis, we supplemented the correlation between its expression and patient prognosis. In addition, Yan found that LINC01305 regulates invasion and migration of CC cells. A series of tests conducted in this study demonstrated that cell proliferation, invasion, and migration were suppressed and apoptosis was enhanced by knocking down si-
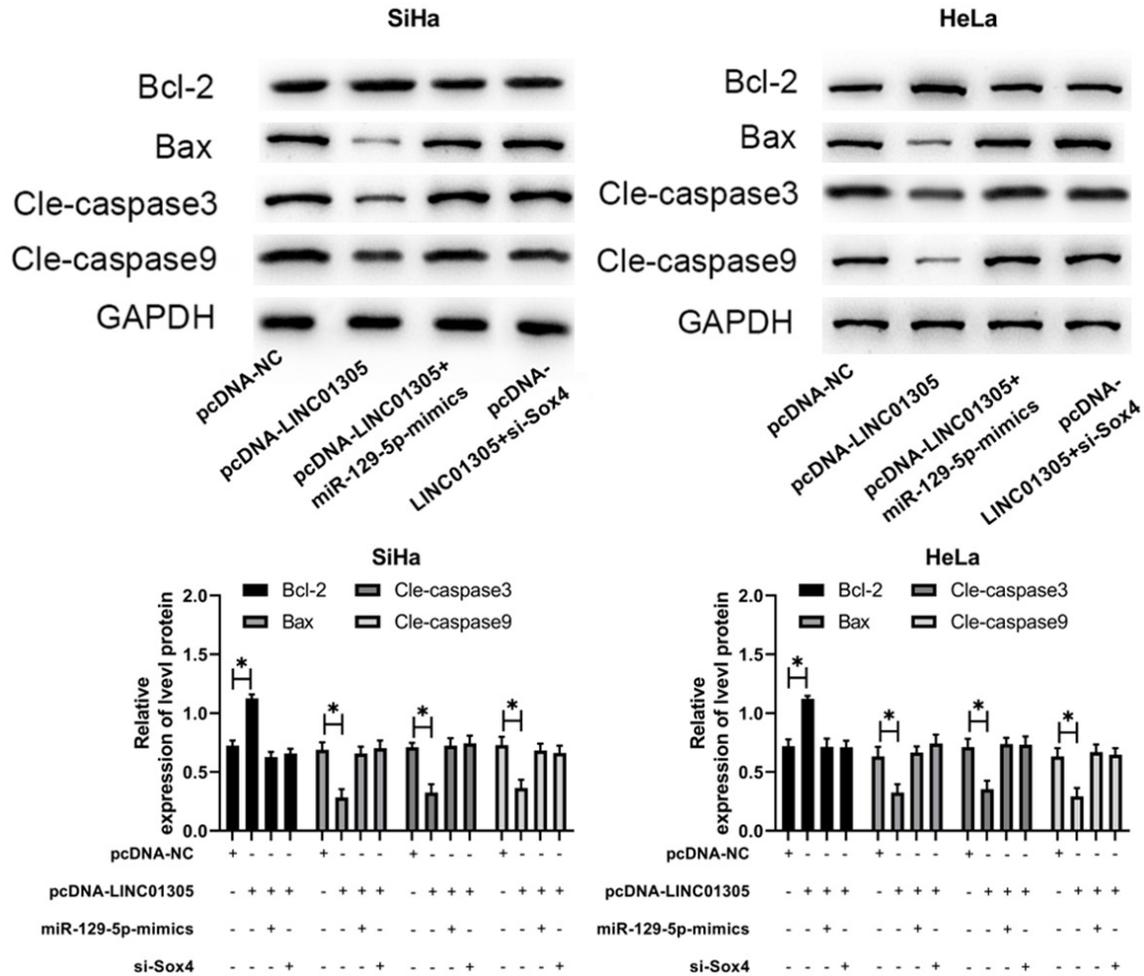
Figure 7. Levels of apoptosis-related proteins after co-transfection are tested by WB. *P < 0.05. WB: Western blot.

LINC01305, indicating the involvement of LINC01305 in the growth of CC cells.

LncRNAs act as sponges to play the role of regulating miRs [28]. After prediction, a close association between LINC01305 and miR-129-5p was revealed. miR-129-5p was lowly expressed in CC and reversely associated with LINC01305, so miR-129-5p might be mediated by LINC01305. And our hypothesis was verified by RIP and DLR assays. miR-129-5p was inhibited in cells treated with si-LINC01305#1, suggesting that LINC01305 can function as a miR-129-5p sponge.

miRs have been reported to participate in tumorigenesis by regulating downstream target genes [29-31]. To figure out the mechanism of miR-129-5p in CC, 5 online prediction websites were employed and found that miR-129-5p

shared targeted binding sites with Sox4. As a member of Sox family, Sox-4 has the function of regulating embryonic development and cell growth [32]. Highly expressed Sox4 promotes CC progression and drug resistance to chemotherapy through ABCG2 [33]. We also found a high expression of Sox4 in CC, and it was positively associated with LINC01305 and reversely associated with miR-129-5p. Moreover, DLR showed us the regulation of miR-129-5p on Sox4. Therefore, we speculated that LINC01305 may be involved in CC by mediating miR-129-5p/Sox4 axis. Malignant phenotypes of cells co-transfected with pcDNA-LINC013-05 and miR-129-5p-mimics or si-Sox4 were enhanced and apoptosis was reduced compared with those treated with miR-129-5p-mimics or si-Sox4 alone. Besides, Bcl-2 increased and Bax, Cle-caspase3 and Cle-caspase9 decreased after co-transfection. Therefore,
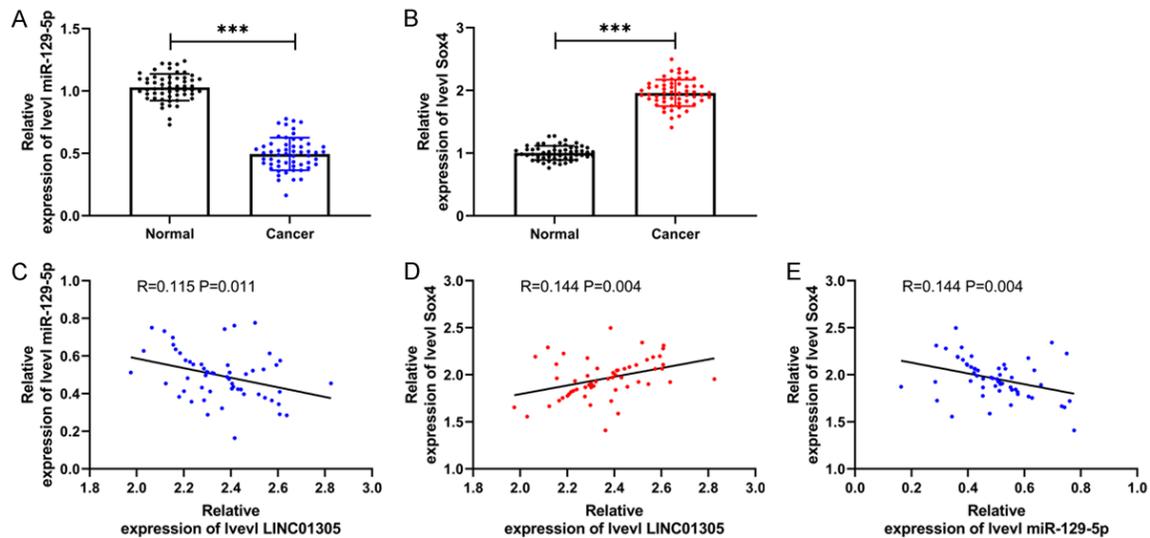
Figure 8. Expression of miR-129-5p and Sox4 in CC and their correlation. A. Relative expression of miR-129-5p in CC is quantified by qRT-PCR. B. Relative expression of Sox4 in CC is quantified by qRT-PCR. C. Correlation between LINC01305 and miR-129-5p in CC is identified by Pearson test. D. Correlation between LINC01305 and Sox4 in CC is identified by Pearson test. E. Correlation between miR-129-5p and Sox4 in CC is identified by Pearson test. ***P < 0.001. CC: cervical cancer; qRT-PCR: quantitative reverse transcription-PCR.

LINC01305 participates in growth and metastasis of CC cells via miR-129-5p/Sox4 axis.

We preliminarily confirmed the potential mechanism of LINC01305 in CC. However, there are still limitations. Firstly, whether LINC01305/miR-129-5p/Sox4 axis has the same regulatory effect *in vivo* is not investigated. Secondly, the implications of lncRNAs in the diagnosis of CC has been increasingly recognized, so further investigation is required to validate the role of LINC01305 in CC diagnosis. We hope to add more tests and include diversified samples to supplement our conclusions.

To sum up, LINC01305 is highly expressed in CC and induces tumorigenesis via miR-129-5p/Sox4 axis. Therefore, it is a promising target for the treatment of CC.

Disclosure of conflict of interest

None.

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