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# LncRNA-CTS promotes metastasis and epithelial-to-mesenchymal transition through regulating miR-505/ZEB2 axis in cervical cancer

Shujun Feng[a], Wei Liu[b], Xiaoxu Bai[b], Wenjing Pan[b], Zhaoyang Jia[b], Shunjin Zhang[b], Yimin Zhu[a], Wenhua Tan[b,*]

[a] Department of Reproductive Endocrinology, Women's Hospital, School of Medicine, Zhejiang University , Xueshi Road NO.1, Hangzhou, Zhejiang, 310000, China
[b] Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Harbin Medical University, Xuefu Road NO.246, Harbin, Heilongjiang, 150001, China

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ABSTRACT

Cervical carcinoma (CC) is the second most common cancer in females. In order to improve current anti-metastasis strategies for CC, it is important to improve our understanding of the mechanisms involved in epithelial-to-mesenchymal transition (EMT). This study aimed to elucidate the potential role of a novel long non-coding RNA (lncRNA)-CTS and the mechanisms underlying EMT in CC. The expression levels of lncRNA-CTS and miR-505 were detected using quantitative reverse transcriptase polymerase chain reaction in CC specimens and cells (HeLa, SiHa, Ca-Ski, C-33A, and HT-3). Further experiments including wound scratch and transwell invasion assays, Western blotting, immunofluorescence, and luciferase assays were used to investigate the function of lncRNA-CTS/miR-505/ZEB2 *in vitro*. In addition, a tumor xenograft model was used to assess the effect of lncRNA-CTS *in vivo*. The expression levels of lncRNA-CTS and miR-505 were correlated with the metastasis-associated clinicopathological features of CC patients. Moreover, lncRNA-CTS was associated with a poor prognosis in CC patients. *In vitro* and *in vivo* experiments, along with gain- and loss-of-function studies, showed that lncRNA-CTS enhanced cell migration, invasion, and the transforming growth factor (TGF)-β1-induced-EMT process. Data also showed that lncRNA-CTS could function as a competing endogenous RNA for miR-505 in CC cells. Further investigations disclosed that ZEB2 was demonstrated as a downstream target of miR-505, and subsequently exerted its metastatic effects via the lncRNA-CTS/miR-505/ZEB2 axis in CC cells. Finally, lncRNA-CTS activated the SMAD/TGF pathway via miR-505 in CC cells. Collectively, our results demonstrate the importance of the lncRNA-CTS/miR-505/ZEB2 axis in CC. LncRNA-CTS can predispose CC patients to metastases and may represent a promising therapeutic target for CC.

## 1. Introduction

Globally, cervical carcinoma (CC) is the second most common cancer in females, accounting for 528,000 new cases and 266,000 deaths worldwide annually [1,2]. However, metastatic CC is incurable, and there is an urgent need to develop novel therapeutic approaches [3]. Current research is especially focused upon elucidating the molecular mechanisms underlying the development of CC and identifying novel molecular targets.

The epithelial-mesenchymal transition (EMT) plays two critical roles in CC. First, EMT changes the cellular characteristics of cancer cells from an epithelial phenotype to a mesenchymal phenotype. Second, EMT increases the ability of cells to invade and migrate, thus promoting metastasis [4,5]. Long non-coding RNAs (lncRNAs) are endogenous RNAs more than 200 nucleotides in length that do not encode proteins. A growing body of evidence now supports the role of lncRNAs in the regulation of gene expression and the activation or repression of EMT process [6,7]. For example, Liang et al. reported that lncRNA-PTAF clearly controlled EMT in the development and progression of ovarian cancer [8]. LncRNA HOST2 has also been reported to promote EMT and enhance metastasis of hepatocellular carcinoma (HCC) cells [9].

Research has already indicated that lncRNA acts as a competing endogenous RNA (ceRNA) to regulate the functions of miRNAs in a range of cancer types [10,11]. LncRNA C5orf66-AS1 was also shown to act as a ceRNA by adsorbing miR-637, thereby regulating the effect of

* Corresponding author. Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Harbin Medical University, Xuefu Road N0. 246, Harbin, Heilongjiang, 150001, China.
*E-mail addresses:* fsj1-1@163.com (S. Feng), liuwei1978@163.com (W. Liu), Baixxx@163.com (X. Bai), pwj1986@163.com (W. Pan), jiazhaoyang@163.com (Z. Jia), shunjin@163.com (S. Zhang), zhuyimin@163.com (Y. Zhu), 13766800936@139.com (W. Tan).

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**Table 1**
Primers used for reverse transcription and PCR in this study.

| Gene | Primer sequence (Forward) | Primer sequence (Reverse) |
|---|---|---|
| lncRNA-CTS | 5′-ACCAGCCCTCACCAAGTTAC-3′ | 5′-GTGTCTTTAGGGCTCTCTTGG-3′ |
| E-cadherin | 5′-GACAACAAGCCCGAATT-3′ | 5′-GGAAACTCTCTCGGTCCA-3′ |
| N-cadherin | 5′-CGGGTAATCCTCCCAAATCA-3′ | 5′-CTTTATCCCGGCGTTTCATC-3′ |
| Vimentin | 5′-GAGAACTTTGCCGTTGAAGC-3′ | 5′-GCTTCCTGTAGGTGGCAATC-3′ |
| miR-505-5p-RT | 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACATCA-3′ | |
| miR-505–5p | 5′-GCGGGAGCCAGGAAGTAT-3′ | 5′-CAGTGCGTGTCGTGGAGT-3′ |
| ZEB2 | 5′-GATGAAATAAGGGAGGGTGG – 3′ | 5′-CCTCAAAATCTGATGTGCAA – 3′ |
| GAPHA | 5′-CATGTTCGTCATGGGTGTGAACCA-3′ | 5′-AGTGATGGCATGGACTGTGGTCAT-3′ |
| U6-RT | 5′-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG-3′ | |
| U6 | 5′-ATTGGAACGATACAGAGAAGATT-3′ | 5′-AGGAACGCTTCACGAATTTG-3′ |

RING1 upon proliferation and apoptosis in CC [12].

LncRNA NR_038940.1 is a noncoding RNA located on chromosome 10 and is 2023 nucleotides in length. In the present study, we report the identification of lncRNA NR_038940.1, which we named lncRNA cancer-associated by transforming growth factor (TGF)-β stimulation (lncRNA-CTS). The data described herein provide evidence for the oncogenic role of lncRNA-CTS, which may represent a novel marker of invasion for patients with CC. Collectively, our data provide new insight for the role of the lncRNA-CTS/miR-505/ZEB2 axis in CC. Our discovery also provides a theoretical basis for the prevention and treatment of CC.

## 2. Materials and methods

### 2.1. Human tissues

Specimens of cervical cancer tissues and normal cervical tissues were obtained from patients undergoing surgery at the Second Affiliated Hospital of Harbin Medical University (Heilongjiang Province, China). The ethics committee of The Second Affiliated Hospital of Harbin Medical University approved this research project (approval No. KY: 2017–032), and informed signed consent was obtained from each patient. The patients diagnosed with cervical cancer and with other non-cancerous gynecological diseases at the Second Affiliated Hospital of Harbin Medical University were included in this study. Only specimens from patients with newly diagnosed cervical cancer, who had not received prior treatment, were included in the study. All tissues were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Clinical and pathological data, including age, International Federation of Gynecology and Obstetrics (FIGO) stage, tumor size, and lymph node status were retrieved from patient records.

### 2.2. Cell culture and transfection

Five CC cell lines (HeLa, SiHa, Ca-Ski, C-33A, and HT-3) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (HyClone, USA) supplemented with 5% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) and 1% penicillin/streptomycin (Beyotime, China). Cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. Only cells with a passage number of less than 20 were used for experiments.

Cells were transfected using Lipofectamine 2000 Reagent (Invitrogen, USA). Opti-MEM (Invitrogen, USA), siRNA-control (si-NC), and lncRNA-CTS siRNA (si-lncRNA-CTS) were purchased from GenePharma (Shanghai, China). An miR-505 inhibitor, a negative control inhibitor (NC inhibitor), a miR-505 mimic (miR-505), and a negative control mimic (miR-NC) were purchased from RiboBio (Guangzhou, China). In order to overexpress lncRNA-CTS and ZEB2, we amplified the full-length lncRNA-CTS and ZEB2 cDNA by polymerase chain reaction (PCR) and sub-cloned these sequences into the pcDNA3.1 plasmid (Invitrogen, USA). After transfection, the culture medium was removed and replaced with serum-free medium; the cells were then incubated for a further 6–8 h, and TGF-β1 (Sigma, USA) was added at a concentration of 10 ng/ml for 48 h.

### 2.3. RNA extraction and quantitative reverse transcriptase-PCR (qRT-PCR)

Total RNA was isolated from cultured cells and tissues using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The total RNA was then reverse transcribed into cDNA using the cDNA reverse transcription kit (TOYOBO, Japan) in accordance with the manufacturer's protocol. The expression levels of mRNA and miRNA were then quantified using a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with a SYBR green PCR Master Mix (TOYOBO, Japan). Fold-changes in relative gene expression were then calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to U6 or GAPDH expression. Table 1 shows the primers used for reverse transcription and PCR.

### 2.4. Cell migration assay

Sterile plastic 10 μL micropipette tips were used to scratch a layer of confluent cells and thus create a linear wound. The cells were then washed three times with phosphate buffer saline (PBS), and the medium was changed to FBS-free DMEM; this was then followed by further incubation for 48 h at 37 °C. Photographic images of cells were captured by light microscopy, at 40 × magnification, at timepoint zero (0 h), and 48 h after wounding.

### 2.5. Cell invasion assay

In order to carry out invasion assays, we coated the upper chambers of a transwell plate (Corning, USA) with Matrigel (1:8, BD Biosciences, CA). Then, 24 h after transfection, $2 \times 10^5$–$4 \times 10^5$ cells (consistent between groups and based on different experiments) were resuspended in 140 μL of FBS-free culture medium and seeded into the top chamber of a transwell plate coated with Matrigel. The bottom chamber was supplemented with complete medium containing 10% FBS. After 48 h of incubation, the cells which had migrated to the other side of the transwell plate were fixed with formaldehyde (Biosharp, South Korea) and stained with hematoxylin and eosin (Solarbio, China). Invading cells in the lower chambers were photographed at 100 × magnification.

### 2.6. Luciferase reporter assay

Sequences of the ZEB2 3′UTR or lncRNA-CTS, containing wild-type or mutated miR-505 binding sites, were synthesized by GenePharma (Shanghai, China). The luciferase vector was co-transfected with the miR-505 mimic or miR-NC into HeLa and SiHa cells using

Lipofectamine 2000 (Invitrogen, USA). As an internal control, Renilla luciferase reporter vector (Promega, USA) were also included. After 48 h of transfection, the cells were collected, and luciferase activity was measured using a luminometer (Titertek-Berthold, USA).

### 2.7. Western blotting

In order to extract protein content, cells were washed twice with cold PBS 72 h after transfection and lysed with radio immunoprecipitation buffer (Thermo Fisher Scientific, USA) at 4 °C for 5 min. Lysates were then centrifuged (4 °C, 15000 rap/min, and 10 min), and the total protein concentration was quantified using the Bicinchoninic acid Protein Assay Reagent Kit (Beyotime, China). For each sample, an equal amount of protein (80 μg) was separated by 8–10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane which was then blocked in 5% skimmed milk and incubated at room temperature for 1 h. After this, the membranes were incubated overnight at 4 °C with a selection of primary antibodies: Vimentin (1:1000, Abcam, USA), N-cadherin (1: 500, Abcam, USA), E-cadherin (1:500, Cell Signaling Technology, USA), ZEB2 (1:800, Abcam, USA), p-SMAD2 (1:1000, Technology, USA), p-SMAD3 (1:1000, Cell Signaling Technology, USA), SMAD2 (1:1000, Cell Signaling Technology, USA), SMAD3 (1:1000, Cell Signaling Technology, USA), and β-actin (1:1000, ZSGB-BIO, China). The following morning, the membranes were incubated with anti-mouse or anti-rabbit secondary antibodies (1:10000; LI-COR Biosciences at room temperature for 1 h). Then, the membranes were washed three times with Tris-buffered saline Tween at room temperature, for 15 min each time. Positive immunoreactivity was then detected using the Odyssey Infrared Imaging System (Gene Company Limited, Hong Kong, China).

### 2.8. RNA-binding protein immunoprecipitation (RIP) assay

HeLa and SiHa cells, which had been transfected with miR-505 mimics or mimics-NC, were lysed and collected in a RIP lysis buffer kit (Millipore, USA). Human anti-$AgO_2$ (Millipore, USA) or mouse anti-IgG (Millipore, USA) were then conjugated with RNAs magnetic beads. qRT-PCR was then used to determine the expression levels of purified RNA.

### 2.9. Immunofluorescence

The cells were seeded into 24-well plates, fixed in 4% paraformaldehyde (Biosharp, South Korea) solution, and permeabilized with 0.1% Triton X-100 (Santa Cruz Biotechnology, USA). The cells were then blocked with 10% BSA (Amersco, USA) for 30 min and then incubated overnight with a primary antibody: anti- SMAD2/3 (1:500; Cell Signaling Technology, USA) at 4 °C. The following morning, the cells were incubated with a FITC-conjugated anti-rabbit secondary antibody (FITC, Invitrogen, CA) for 1 h at 4 °C. Next, the cells were stained with diamidino-2-phenylindole (DAPI; 1:1000; Beyotime, China) for 15 min, and photographic images were acquired with confocal microscopy (OLYMPUS, Japan).

### 2.10. Tumor xenografts in animals

Lentiviruses containing siRNA for lncRNA-CTS, or scramble lentiviruses, were purchased from GenePharma Company (Shanghai, China). These lentiviruses were mixed directly with HeLa cells at a 20:1 multiplicity-of-infection ratio, followed by selection with 1 μg/mL puromycin for 14 days. Four-week-old female BALB/c nude mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All of the BALB/c nude mice were maintained under Specific Pathogen Free conditions. Stably transfected HeLa cells ($5 \times 10^6$/0.2 ml in PBS) were implanted into intraperitoneal of nude mice. TGF-β1 was injected into tail vein of nude mice every 3 days. After 35 days, the mice were sacrificed by spinal cord amputation, and the weights and volumes of tumors were measured and recorded. Then, the lungs, liver, colorectum, and abdominal lymph nodes of each mouse were assessed; macroscopic metastatic foci were counted, collected, and photographed. All the metastatic tissues were examined histopathologically, except inflammatory diseases and physiological tissue proliferation. The protocols for these animal experiments were approved by the Ethics Review Committee of the Second Affiliated Hospital of Harbin Medical University.

### 2.11. Statistical analysis

Statistical analysis was carried out using SPSS 22.0 (Inc., Chicago, IL, USA) and GraphPad V6.0 (Inc., La Jolla, CA, USA) software. All experiments were performed in triplicate, and the data is presented as the mean plus or minus the standard deviation (SD). The statistical approaches mainly included a two-tailed Student's *t*-test, analysis of variance, a Kaplan–Meier plot, Pearson chi-squared test, and Pearson's correlation analysis. A two-tailed Student's *t*-test was used to compare differences between the two groups, and analysis of variance was applied to compare differences between more than two groups. A Kaplan-Meier plot was used for survival curves. The Pearson chi-squared test was used to analyze the relationship between clinicopathological characteristics and the expression of lncRNA-CTS or miR-505. Pearson's correlation test was used to analyze the correlation between lncRNA-CTS and miR-505 expression. Instances where $P < 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. LncRNA-CTS is expressed at high levels in CC tissues

To identify differentially expressed lncRNAs in CC, we analyzed high-throughput lncRNA microarray data from the publicly available GENE EXPRESSION OMNIBUS (GEO) database [14]. A heatmap showed that there were 203 dysregulated lncRNAs in CC tissues (all $P < 0.05$; > 3.0 fold). We chose 15 lncRNAs in which the fold change was > 5.0 and determined their expression in 10 CC tissues by qRT-PCR. As shown in Fig. 1B, our analysis highlighted lncRNA NR_038940.1, which showed the most significant upregulation in CC tissues. Furthermore, analysis of another GEO dataset (Reference number: GSE26511) showed that the expression of lncRNA-CTS was higher in CC patients with lymph node metastasis, compared to patients with no lymph node spread ($P = 0.014$; Fig. 1C).

Furthermore, qRT-PCR was used to determine the expression levels of lncRNA-CTS in 50 specimens of CC and 50 cervical tissues proven to be histologically normal. LncRNA-CTS levels were significantly higher in tumor tissues ($P = 0.007$; Fig. 1D). The expression of lncRNA-CTS was upregulated in FIGO stages III-IV ($P = 0.004$; Fig. S1A) and was associated with a poor histology grade ($P = 0.006$; Figs. S1B) and a larger tumor size in CC ($P = 0.020$; Fig. S1C).

Patients were then sub-classified into high-lncRNA-CTS and low-lncRNA-CTS groups according to lncRNA-CTS expression in CC tissues. This allowed us to further investigate the precise relationships between lncRNA-CTS expression and clinical pathological features. Our findings showed that the upregulation of lncRNA-CTS was correlated with poor tumor histology grade ($P = 0.009$), large tumor size ($P = 0.023$), FIGO stage ($P = 0.002$), and lymph node metastasis ($P = 0.023$) in CC. However, lncRNA-CTS expression was not correlated with patient age or serum levels of squamous cell carcinoma (SCC; Table 2). Next, we used qRT-PCR to investigate the expression levels of lncRNA-CTS in five CC cell lines and one normal human cervical epithelial cell line Ect1/E6E7. LncRNA-CTS was upregulated in five CC cell lines (HeLa, SiHa, Ca-Ski, C-33A, and HT-3) compared with Ect1/E6E7 ($P = 0.012$, $P = 0.003$, $P = 0.023$, $P = 0.002$, and $P = 0.031$, respectively; Fig. 1E). Among the five CC cells, HeLa cells showed the highest expression level

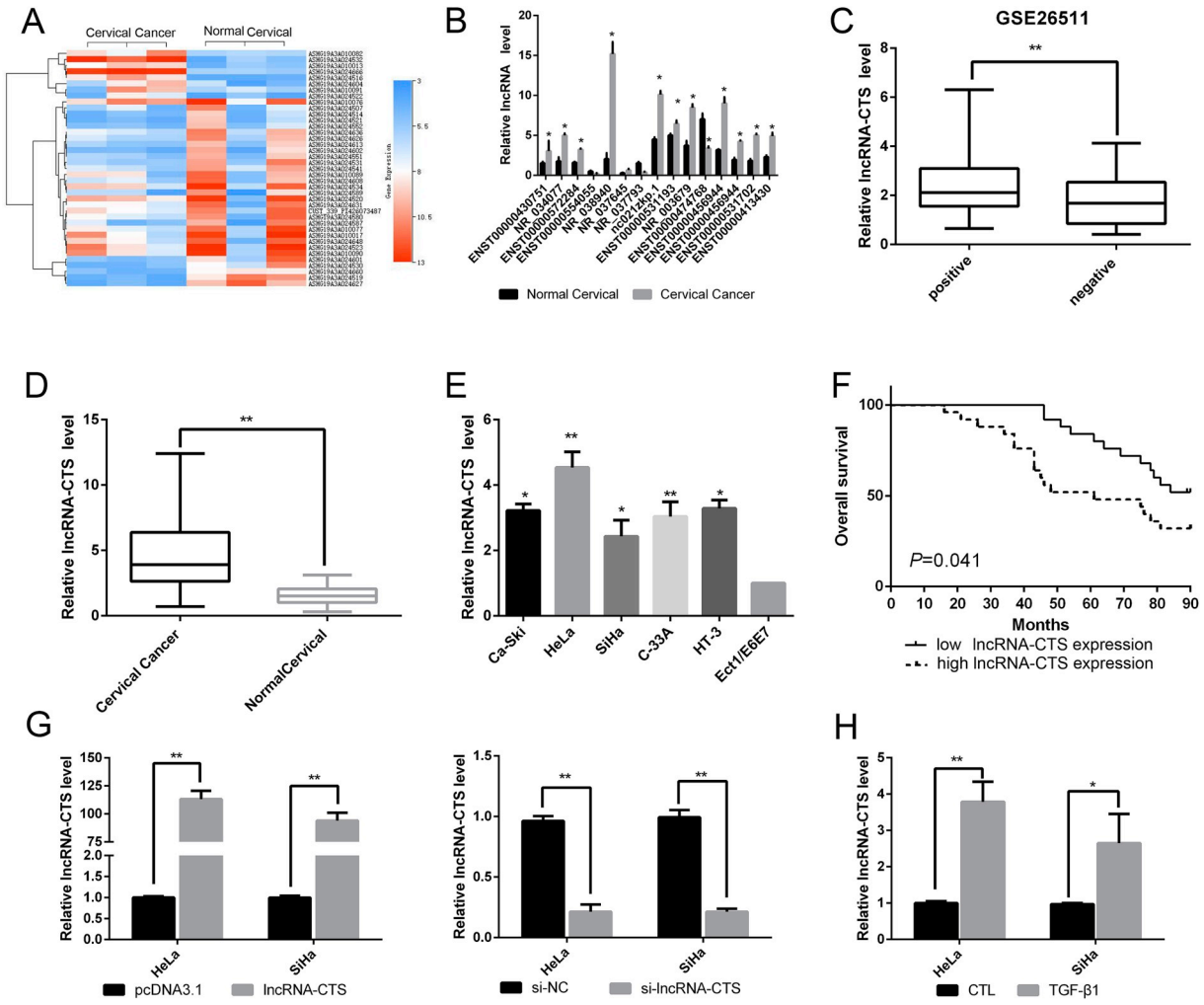


**Fig. 1.** Levels of LncRNA-CTS were clearly upregulated in tissues and cells with CC. **(a)** A heatmap showing differentially expressed lncRNAs (all $P < 0.05$; > 3-fold) between CC tissues (n = 3) and cervical tissues (n = 3). **(b)** Expression levels of 15 lncRNAs in CC tissues (n = 10) and normal cervical tissues (n = 10), as assessed by qRT-PCR assay. **(c)** GEO dataset (GSE26511) from R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) indicated that lncRNA-CTS expression was higher in CC patients with lymph node metastasis, compared to patients with no lymph node spread. **(d)** Expression levels of lncRNA-CTS in CC tissues (n = 50) and normal cervical tissues (n = 50), as assessed by qRT-PCR assay. **(e)** LncRNA-CTS expression in five CC cell lines and one non-carcinoma cervical epithelial human papillomavirus-16 E6/E7 transformed cell line. **(f)** The relationship between lncRNA-CTS expression and the overall survival of CC patients. **(g)** Validation of si-lncRNA-CTS and pcDNA3.1-lncRNA-CTS efficiency in CC cells, as determined by qRT-PCR. **(h)** Expression of lncRNA-CTS in HeLa and SiHa cells treated with TGF-β1, as determined by qRT-PCR. The data are presented as mean ± standard deviation (SD) from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

of lncRNA-CTS while SiHa cells showed the lowest expression level of lncRNA-CTS, and these two cells were selected for additional functional studies.

We then evaluated the overall survival records of patients subsequent to surgery. We found that CC patients in the high lncRNA-CTS group were associated with a poorer outcome compared to those in the low lncRNA-CTS group ($P = 0.041$; Fig. 1F). These results implied that lncRNA-CTS might be useful as a novel marker of CC progression.

### 3.2. LncRNA-CTS enhances TGF-β1-induced cell migration, invasion and EMT

To further investigate the role of lncRNA-CTS in the tumorigenesis of CC, we constructed a recombinant pcDNA3.1 plasmid to upregulate lncRNA-CTS and siRNA constructs to knockdown lncRNA-CTS. Transfection efficiency was measured by a qRT-PCR assay ($P < 0.01$; Fig. 1G). The overexpression of lncRNA-CTS was significantly upregulated in both HeLa and SiHa cells in response to 10 ng/ml of multifunctional cytokine TGF-β1 over 48 h ($P < 0.01$; Fig. 1H). TGF-β1 orchestrates an intricate signaling network to modulate tumorigenesis and progression and promotes tumor progression by enhancing proliferation, migration, and invasion, in part by its ability to induce EMT [13]. As shown in Supplementary Fig. 2A–B, the knockdown of lncRNA-CTS inhibited the expression of TGF-β1 ($P < 0.05$). In contrast, the overexpression of lncRNA-CTS promoted the expression of TGF-β1 in HeLa and SiHa cells ($P < 0.05$; Figs. S2C–D). Combined with our previous results, these data suggest that there is a positive feedback loop between TGF-β1 and lncRNA-CTS. Therefore, we speculated that lncRNA-CTS may contribute to TGF-β1-mediated EMT in CC cells, along with migration and invasion.

Wound healing and transwell invasion assays showed that the knockdown of lncRNA-CTS reduced the capacity for migration and invasion in HeLa and SiHa cells. Furthermore, this effect was restored by treating the cells with TGF-β1 (10 ng/ml; $P < 0.05$; Fig. 2A–D). In contrast, the migration and invasion capacity increased following the overexpression of lncRNA-CTS in both HeLa and SiHa cells; TGF-β1 treatment could promote this process ($P < 0.05$; Fig. 2G–J).

The levels of the epithelial marker E-cadherin were increased while levels of the mesenchymal markers, Vimentin and N-cadherin, were downregulated following the transfection of HeLa and SiHa cells with

**Table 2**
Correlation between the clinicopathologic characteristics lncRNA-CTS and miR-505 expression in CC(n = 50).

| Clinical parameters | Expression Level | | *P* value | Expression Level | | *P* value |
|---|---|---|---|---|---|---|
| | lncRNA-CTS$^{High}$ | lncRNA-CTS$^{Low}$ | | miR-505$^{High}$ | miR-505$^{Low}$ | |
| **Age(year)** | | | | | | |
| ≥50 | 11 | 16 | 0.156 | 12 | 15 | 0.395 |
| < 50 | 14 | 9 | | 13 | 10 | |
| **FIGO stage** | | | | | | |
| III-IV | 19 | 8 | 0.002** | 10 | 17 | 0.047* |
| I-II | 6 | 17 | | 15 | 8 | |
| **Tumor Size(cm)** | | | | | | |
| ≥4 | 17 | 9 | 0.023* | 9 | 17 | 0.024* |
| < 4 | 8 | 16 | | 16 | 8 | |
| **Lymph node metastasis** | | | | | | |
| Yes | 18 | 10 | 0.023* | 5 | 23 | < 0.001** |
| No | 7 | 15 | | 20 | 2 | |
| **Histology grade** | | | | | | |
| Poor | 14 | 5 | 0.009** | 4 | 15 | 0.001** |
| Well and moderate | 11 | 20 | | 21 | 10 | |
| **Serum SCC level** | | | | | | |
| ≥1.5 ng/mL | 15 | 19 | 0.225 | 18 | 16 | 0.544 |
| < 1.5 ng/mL | 10 | 6 | | 7 | 9 | |

si-lncRNA-CTS. The administration of TGF-β1 induced the downregulation of E-cadherin and the upregulation of N-cadherin and Vimentin. In addition, the effects of TGF-β1 upon the levels of these EMT markers were significantly attenuated by the knockdown of lncRNA-CTS in HeLa and SiHa cells ($P < 0.05$; Fig. 2E and F). Consistent with these results, the overexpression of lncRNA-CTS promoted the EMT process in HeLa and SiHa cells; TGF-β1 treatment was found to promote this effect ($P < 0.05$; Fig. 2K-L).

Collectively, these data suggest that lncRNA-CTS is required for TGF-β1-induced EMT, cell migration, and invasion in CC.

### 3.3. LncRNA-CTS acts as a molecular sponge for miR-505 in CC

Using both the bioinformatics tool, Regrna2.0 (http://regrna2.mbc.nctu.edu.tw/), and the qRT-PCR data, miR-505 exhibited the greatest change in HeLa and SiHa cells following transfection with si-lncRNA-CTS or lncRNA-CTS.

qRT-PCR results showed that the expression level of miR-505 in 50 CC specimens was significantly lower than in 50 normal cervical tissues ($P < 0.01$; Fig. 3A). Pearson correlation analysis revealed a negative association between lncRNA-CTS and miR-505 expression in CC tissues ($r = -0.288$; $P = 0.042$; Fig. 3B). HeLa and SiHa cells were transfected with miR-505 inhibitors and mimics, respectively, and transfection efficiency was measured by qRT-PCR assay ($P < 0.05$; Fig. 3C and D). The expression levels of miR-505 and lncRNA-CTS were negatively correlated in HeLa and SiHa cells; we also found that TGF-β1 played a role in regulating this process ($P < 0.05$; Fig. 3E–H). In addition, as shown in Fig. 3I, the levels of miR-505 expression were significantly downregulated in both HeLa and SiHa cells in response to treatment with TGF-β1 ($P < 0.01$).

We also constructed luciferase reporter vectors that contained wild-type or mutant miR-505 putative binding sites in lncRNA-CTS (Fig. 3J). As shown in Fig. 3K, the relative luciferase activity in cells co-transfected with lncRNA-CTS-WT and miR-505 mimics was significantly reduced in cells co-transfected with lncRNA-CTS-WT and miR-NC ($P < 0.01$). Furthermore, the RIP assay results showed that miR-505 was a direct target of lncRNA-CTS in CC cells and acted via $AgO_2$ ($P < 0.05$; Fig. 3L–M).

These data suggest that lncRNA-CTS could act as a miRNA sponge for miR-505 by directly binding to complementary sequences in CC cells.

### 3.4. miR-505 inhibits TGF-β1-induced migration, invasion, and EMT

To further explore whether miR-505 exerts influence over TGF-β1-induced migration, invasion, and EMT, we performed wound healing, transwell assays, and Western blotting on HeLa and SiHa cells which had been transiently transfected with miR-505 mimics or miR-NC in the presence or absence of TGF-β1.

Wound healing and transwell assays demonstrated that the overexpression of miR-505 attenuated TGF-β1-induced migration and invasion in both HeLa and SiHa cells ($P < 0.05$; Fig. 4A–D). As shown in Fig. 4E and F, the upregulation of miR-505 inhibited TGF-β1-induced upregulation of N-cadherin and Vimentin and increased the TGF-β1-induced downregulation of E-cadherin in HeLa and SiHa cells ($P < 0.05$). These results suggest that miR-505 is involved in TGF-β1-induced EMT.

To further explore the correlation between miR-505 expression and clinical pathological features, we divided our patients into two groups based on the levels of miR-505 expression in 50 CC specimens. The data showed that miR-505 was correlated with tumor histology grade ($P = 0.031$), tumor size ($P = 0.021$), FIGO stage ($P = 0.011$), and lymph node metastasis ($P = 0.005$) in CC (Table 2).

### 3.5. LncRNA-CTS modulates TGF-β1-induced migration, invasion, and EMT via miR-505

After considering our previous data, we next postulated that lncRNA-CTS modulates TGF-β1-induced migration, invasion, and EMT via miR-505.

Wound healing and transwell assays further showed that lncRNA-CTS knockdown reduced TGF-β1-mediated migration and invasion in CC cells; these effects were abolished following transfection with an inhibitor of miR-505 ($P < 0.05$; Fig. 5A–D). Conversely, our data showed that overexpression of lncRNA-CTS enhanced TGF-β1-induced migration and invasion, but miR-505 restrained migratory and invasive properties of HeLa and SiHa cells ($P < 0.05$; Figs. S3A–D). Next, Western blotting assay indicated that, as in our previous data, depletion of lncRNA-CTS inhibited TGF-β1-induced EMT, which can be abrogated by inhibition of miR-505 ($P < 0.05$; Fig. 5E and F). Ectopic expression of lncRNA-CTS in CC cells promoted TGF-β1-induced EMT, while miR-505 overexpression rescued these effects ($P < 0.05$; Figs. S3E–F).

Taken together, this data suggests the role for lncRNA-CTS in inducing TGF-β1-mediated EMT. This effect depends on the competitive binding of miR-505.

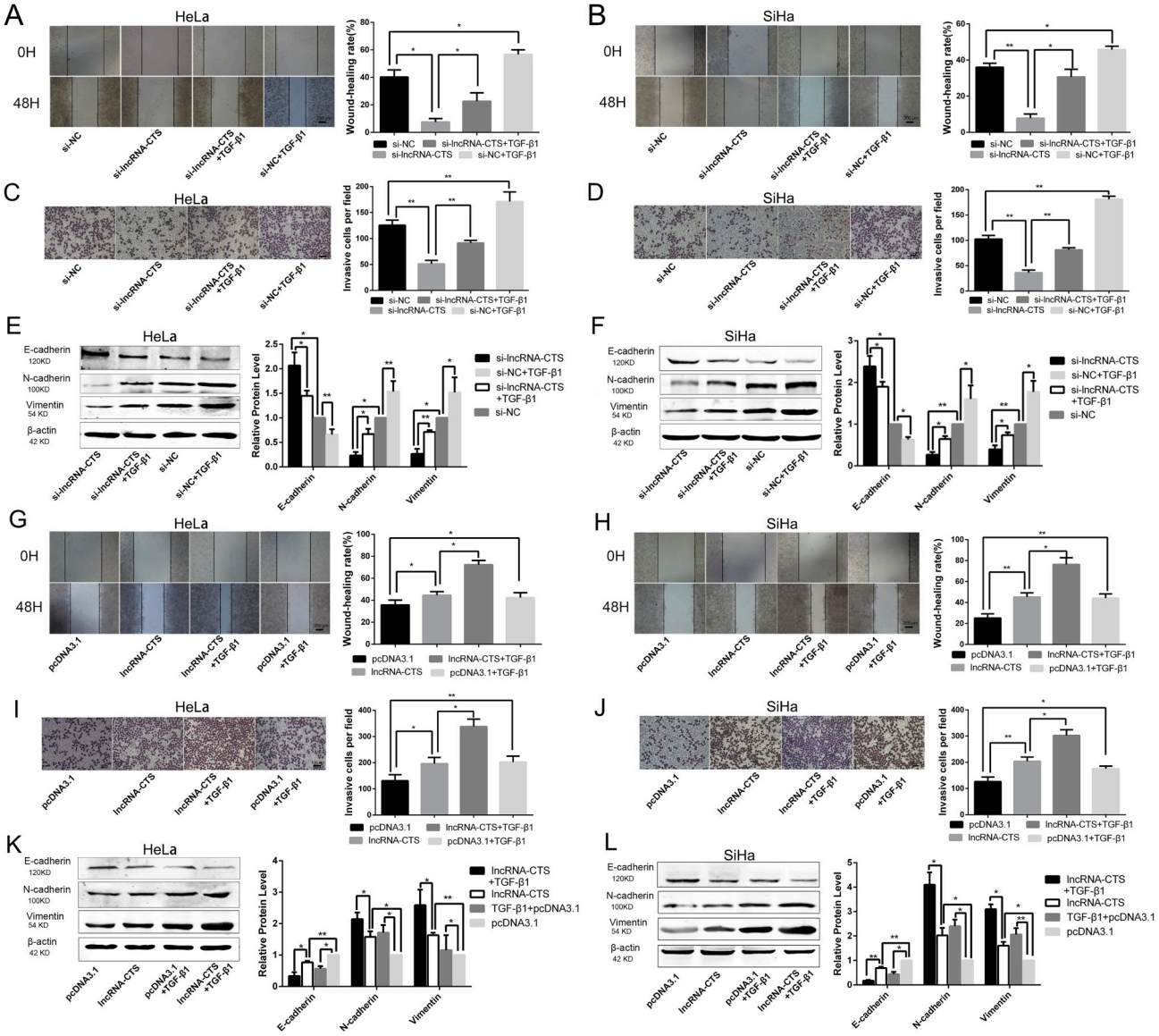


**Fig. 2.** Effects of lncRNA-CTS on migration, invasion, and EMT in CC cells. (**a, b**) Wound assays carried out on HeLa and SiHa cells transfected with si-NC or si-lncRNA-CTS, with or without TGF-β1. (**c, d**) Transwell assays carried out on HeLa and SiHa cells transfected with si-NC or si-lncRNA-CTS, with or without TGF-β1. (**e, f**) Protein levels of EMT markers in HeLa and SiHa cells transfected with si-NC or si-lncRNA-CTS followed by TGF-β1, as determined by Western blotting. (**g, h**) Wound assays carried out on HeLa and SiHa cells transfected with pcDNA3.1 or pcDNA3.1-lncRNA-CTS, with or without TGF-β1. (**i, j**) Transwell assays carried out on HeLa and SiHa cells transfected with pcDNA3.1 or pcDNA3.1-lncRNA-CTS, with or without TGF-β1. (**k, l**) Protein levels of EMT markers in SiHa and HeLa cells transfected with pcDNA3.1 or pcDNA3.1-lncRNA-CTS followed by TGF-β1, as determined by Western blotting. The data are presented as mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

### *3.6. miR-505 exerts influence over TGF-β1-mediated migration, invasion, and EMT by targeting ZEB2*

In order to identify the molecular mechanism by which miR-505 influences the biological functions of CC, we searched for candidate targets of miR-505 on the TargetScan (7.1) database (http://www.targetscan.org). Binding sequences for miR-505 were identified in the 3′UTR of ZEB2 mRNA. Consequently, we postulated that miR-505 could bind directly to its target, ZEB2, in CC cells.

As shown in Fig. 6A and Figs. S4A–B, we also found that the mRNA and protein expression of ZEB2 was negatively regulated by miR-505 in CC cells, which was also influenced by TGF-β1 treatment ($P < 0.05$). Furthermore, we performed luciferase reporter assays and confirmed that ZEB2 binds directly to miR-505. In both HeLa and SiHa cells, miR-505 mimics significantly reduced the relative luciferase activity of ZEB2-WT; however, this reduction was not observed in the ZEB2-MUT group ($P < 0.01$; Fig. 6B). Thus, we proved that ZEB2 was a novel target of miR-505 in CC cells.

Next, we investigated whether miR-505 could exert inhibitory effects upon migration, invasion, and EMT progression in CC by targeting ZEB2. As shown in Fig. 6C–F, wound healing and transwell assays demonstrated that the overexpression of ZEB2 promoted TGF-β1-induced migration and invasion in both HeLa and SiHa cells ($P < 0.05$), which was attenuated by treatment with miR-505 mimics. Interestingly, the invasion ability in SiHa cells was higher in cells treated with TGF-β1, pcDNA3.1-ZEB2, and miR-505 than in cells treated with TGF-β1 alone; in HeLa cells, we detected the opposite results. Increased levels of N-cadherin and Vimentin protein and reduced levels of E-cadherin protein were detected in HeLa cells that had been treated with pcDNA3.1-ZEB2 and TGF-β1 compared to TGF-β1 alone; these effects, which were associated with ZEB2 overexpression, were partially reversed by miR-505 mimics ($P < 0.05$; Fig. 6G). Similar effects were observed in SiHa cells,

but the relative elevation in protein levels were not as pronounced as those in HeLa cells ($P < 0.05$; Fig. 6H).

### 3.7. LncRNA-CTS activates the TGF/SMAD pathway by targeting miR-505

In the present study, Western blotting results showed that the knockdown of lncRNA-CTS led to the decreased expression of p-SMAD2 and p-SMAD3 in both HeLa and SiHa cells, although there was no significant change in the levels of SMAD2 and SMAD3 proteins; these effects were reversed following transfection with miR-505 inhibitor ($P < 0.05$; Fig. 7A and B). Furthermore, the overexpression of lncRNA-CTS led to an increase in the expression levels of p-SMAD2 and p-SMAD3; the co-transfection of lncRNA-CTS and miR-505 reversed the upregulation of p-SMAD2 and p-SMAD3 induced by lncRNA-CTS

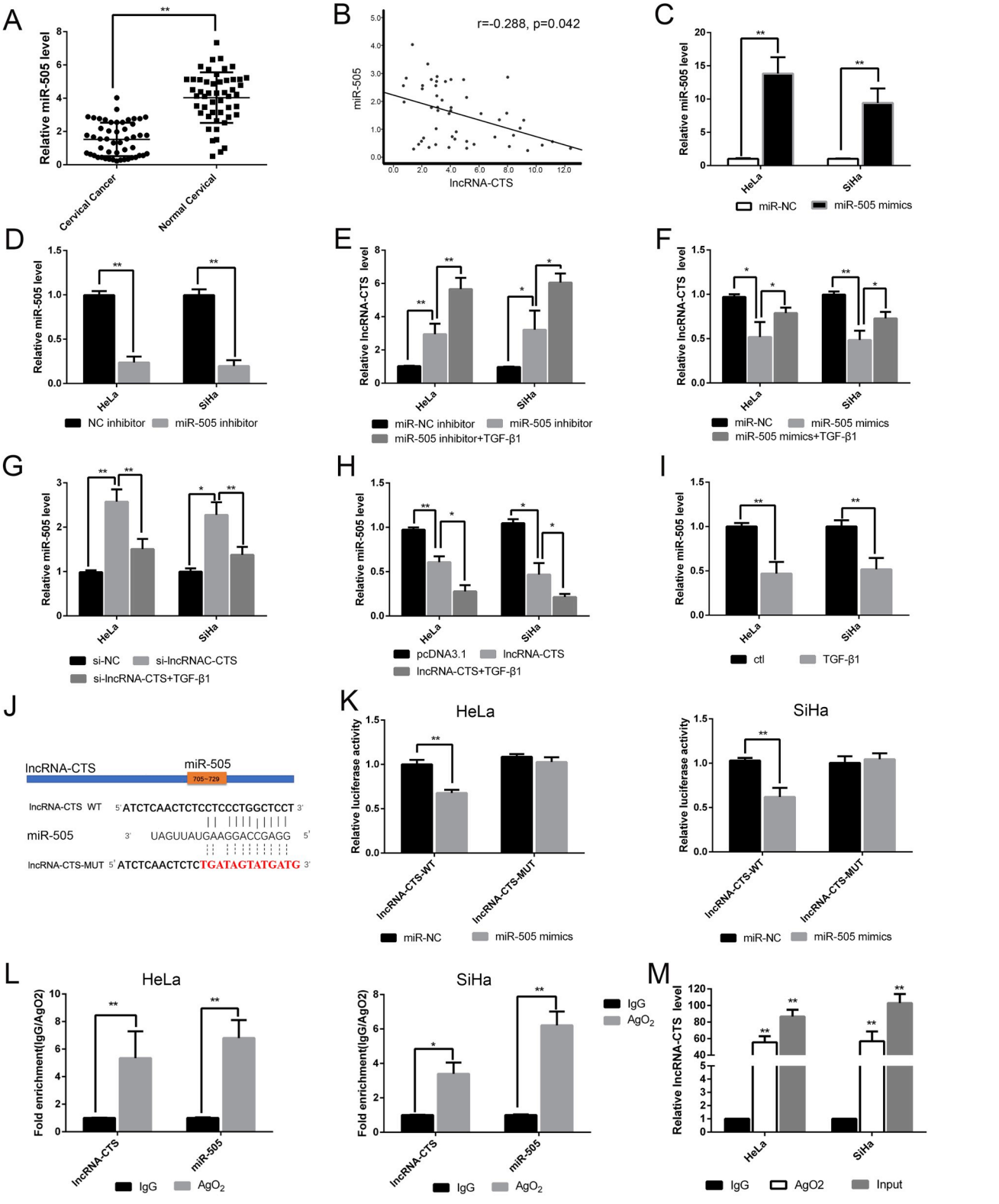


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**Fig. 3.** LncRNA-CTS was shown to negatively regulate the expression and activity of miR-505 in CC cells. (**a**) The expression levels of miR-505 in CC and normal cervical tissues, as assessed by qRT-PCR assay (n = 50). (**b**) The relationship between lncRNA-CTS and miR-505 expression in CC patients. (**c**) Validation of the efficiency of miR-505 mimics in CC cells, as determined by qRT-PCR. (**d**) Validation of the efficiency of miR-505 inhibitor in CC cells, as determined by qRT-PCR. (**e**) Expression of lncRNA-CTS in HeLa and SiHa cells transfected with an miR-505 inhibitor, with or without TGF-β1, as determined by qRT-PCR. (**f**) Expression of lncRNA-CTS in HeLa and SiHa cells transfected with miR-505 mimics, with or without TGF-β1, as determined by qRT-PCR. (**g**) Expression of miR-505 in HeLa and SiHa cells transfected with si-lncRNA-CTS or si-NC, with or without TGF-β1, as determined by qRT-PCR. (**h**) Expression of miR-505 in HeLa and SiHa cells transfected with pcDNA3.1 or pcDNA3.1-lncRNA-CTS, with or without TGF-β1, as determined by qRT-PCR. (**i**) Expression of miR-505 in TGF-β1-treated HeLa and SiHa cells, as determined by qRT-PCR. (**j**) RegRNA2.0 revealed the predicted miR-505 binding site in the lncRNA-CTS 3′UTR. Wildtype (WT) and mutated (MUT) 3′UTRs of lncRNA-CTS are also shown. (**k**) Luciferase reporter activity of chimeric vectors carrying the luciferase gene and a fragment of lncRNA-CTS containing the WT binding site or a mutated binding site for miR-505. (**l**) The correlations between lncRNA-CTS, miR-505 and Ago2 were detected in the RIP assay with anti-Ago2 in CC cells. (**m**) HeLa and SiHa cells with miR-505 mimics or mimics-NC were used in RIP assays. The expression of lncRNA-CTS was also detected by PCR. All data are given as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01.

($P < 0.05$; Fig. 7C and D).

Immunofluorescent data showed that the overexpression of lncRNA-CTS increased the nuclear distribution of SMAD2/3, and miR-505 mimics partly rescued the effects of lncRNA-CTS in SiHa cells ($P < 0.05$; Fig. 7E and F). Treatment with the TGF-β1 receptor antagonist SB431542 had no significant effect on the nuclear distribution of SMAD2/3 after altering the expression of lncRNA-CTS or miR-505 in HeLa and SiHa cells ($P < 0.05$; Fig. 7G and H).

These results indicate that lncRNA-CTS activates TGF/SMAD signaling and that the effect of lncRNA-CTS on TGF/SMAD signaling is dependent on miR-505.

### 3.8. LncRNA-CTS knockdown reduces tumorigenicity and metastasis in vivo

To confirm the role of lncRNA-CTS in cervical cancer metastasis *in vivo*, we generated stable downregulation of lncRNA-CTS expression in HeLa cells by sh-lncRNA-CTS transfection. Macroscopic observation of nude mice revealed that tumor size and weight were lower in the sh-lncRNA-CTS group than the sh-NC group (Fig. 8A). Furthermore, the mean number of harvested nodules was reduced in sh-lncRNA-CTS knockdown mice compared with the sh-NC group ($P < 0.05$; Fig. 8B). Consistent with the results *in vitro*, knockdown of lncRNA-CTS expression could decrease the level of lncRNA-CTS and increase the level of miR-505 in xenograft tumors ($P < 0.05$; Fig. 8C and D).

We also used qRT-PCR and Western blotting to detect expression of E-cadherin, N-cadherin, and Vimentin, in tumor tissues. We observed that the mRNA and protein levels of E-cadherin were increased and accompanied by a reduction in Vimentin and N-cadherin in xenograft tumors from the sh-lncRNA-CTS mice ($P < 0.05$; Fig. 8F). We also measured the expression of ZEB2 by qRT-PCR and Western blotting and detected significantly reduced expression of ZEB2 in the sh-lncRNA-CTS group compared to the sh-NC group ($P < 0.01$; Fig. 8G).

In summary, the knockdown of lncRNA-CTS was sufficient to reduce expression levels of ZEB2 and thus inhibit EMT.

## 4. Discussion

A growing body of evidence suggests that lncRNAs play a key role in the occurrence and progression of tumors [14]. In the present study, we found that lncRNA-CTS promotes TGF-β1-induced EMT by competitively binding to miR-505, upregulating ZEB2, and then activating TGF/SMAD signaling in CC. Therefore, lncRNA-CTS plays a metastatic role in CC (Fig. 8H).

We first demonstrated increased expression levels of lncRNA-CTS in CC, and the higher expression levels of lncRNA-CTS were correlated with a larger tumor size, poorer tumor differentiation, FIGO stage, and lymph node metastasis in CC patients and was correlated with a poorer prognosis. Collectively, this data supports our conclusion that lncRNA-CTS has pleiotropic effect on the metastasis of CC cells. We can therefore conclude that lncRNA-CTS exhibits oncogenic activity in CC.

Metastasis is a multistep process in which tumor cells break away from the primary tumor, translocate, and then form secondary tumors

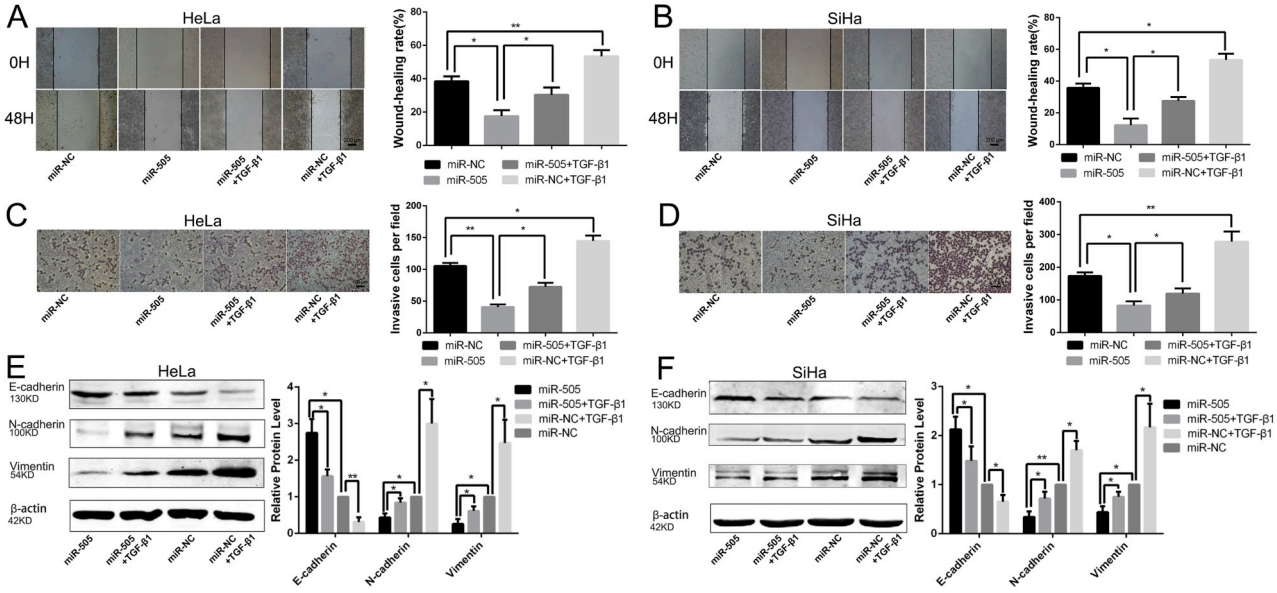


**Fig. 4.** Effects of miR-505 on migration, invasion, and EMT in CC cells. (**a, b**) Wound assays carried out on HeLa and SiHa cells transfected with miR-505 mimics or mimics-NC, with or without TGF-β1. (**c, d**) Transwell assays carried out on HeLa and SiHa cells transfected with miR-505 mimics or mimics-NC, with or without TGF-β1. (**e, f**) Protein levels of EMT markers in SiHa and HeLa cells transfected with miR-505 mimics or mimics-NC followed by TGF-β1, as determined by Western blotting. All data are presented as mean ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$.

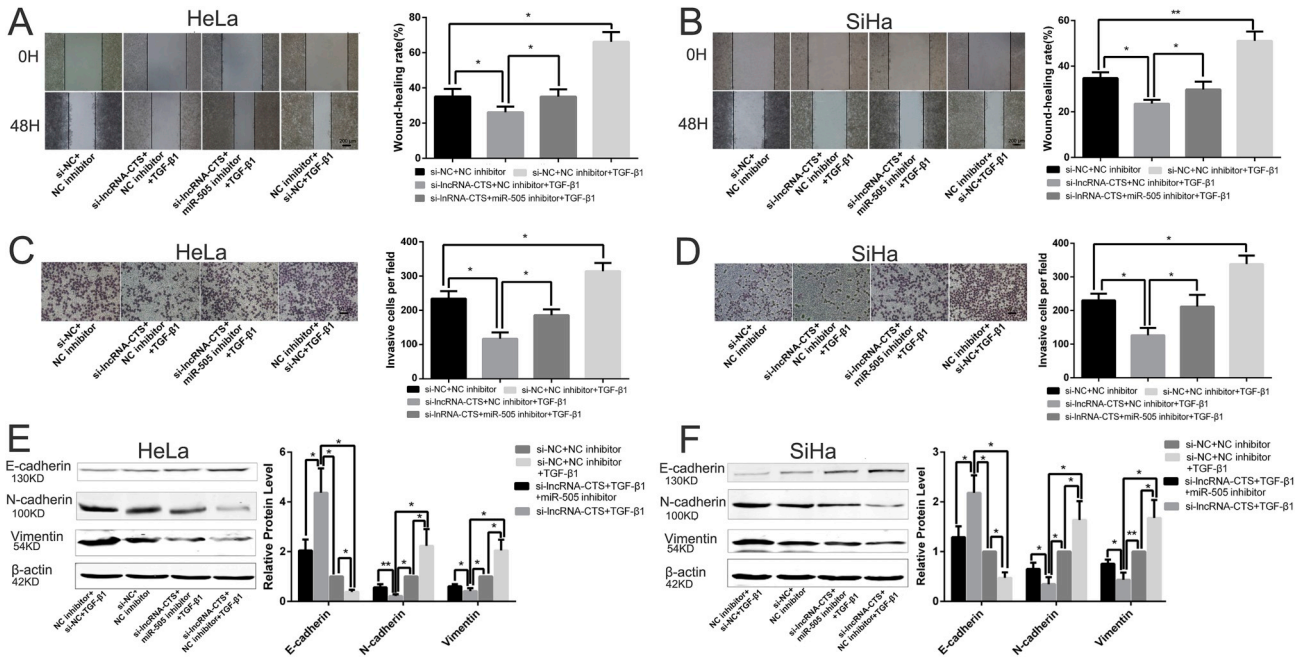


**Fig. 5.** LncRNA-CTS modulates TGF-β1-induced migration, invasion, and EMT via miR-505. (**a, b**) Wound assays were carried out on HeLa and SiHa cells, which had been treated in specific ways. HeLa and SiHa cells were transfected with or without si-lncRNA-CTS and miR-505 inhibitor, in the presence or absence of TGF-β1. (**c, d)** Transwell assays were carried out on HeLa and SiHa cells, which had been treated in specific ways, after 48 h of incubation. (**e, f**) Protein levels of EMT markers in HeLa and SiHa cells, treated as specified and determined by Western blotting assays. All data are presented as mean ± SD from at least three independent experiments. $*P < 0.05$, $**P < 0.01$.

elsewhere [15,16]. The EMT process leads to metastasis and the progression of cancer. TGF-β1 is one of the predominant inducers of metastasis and promotes EMT [17]. Intriguingly, recent studies have shown that lncRNAs also participate in the TGF-β1-induced EMT process. For instance, lncRNA-PTAR was previously shown to promote TGF-β1-induced EMT in ovarian cancer by regulating the expression of ZEB1 via miR-101 in non-small-cell lung cancer (NSCLC) [18]. In another study, lncRNA-ATB, a regulator of TGF-β1 signaling, was shown to competitively bind to members of the miR-200 family, which promoted metastasis and the progression of EMT in HCC [19]. Similar to these earlier studies, we found that lncRNA-CTS promoted TGF-β1-induced EMT, cell migration, and invasion in CC.

LncRNAs are known to control gene expression in a precise manner through a variety of different methods. One most important method is to antagonize the function of miRNAs by competing with miRNAs to bind to shared target mRNAs, which can lead to the silencing of some mRNAs, such as Linc00462 and H19 [20–23]. In this study, bioinformatics analysis, luciferase reporter assay, and RIP assays showed that lncRNA-CTS acted as a ceRNA of miR-505 in CC cells. A previous paper reported that miR-505 functions as a tumor suppressor during the metastasis of CC [24]. As expected, our studies showed that lncRNA-CTS acts as an oncogenic gene by targeting miR-505 in CC. Furthermore, miR-505 regulates gene expression by binding to the 3′UTR of ZEB2.

ZEB2 is a member of the family of zinc finger homeodomain transcription factors and functions as a master molecular switch during the EMT process [25,26]. A range of studies have demonstrated that several lncRNAs can activate the processes involved in TGF-β-induced EMT by modulating ZEB2. Li et al. reported that the knockdown of lncRNA XIST inhibited EMT process that had been stimulated by TGF-β1 in NSCLC via miR-367/miR-141/ZEB2 [27]. Consistent with these earlier results, our current data suggest that the restoration of ZEB2 abolished the effects of miR-505 on TGF-β1-induced-EMT process in CC cells. It is important to highlight the fact that short-term TGF-β1 treatment is sufficient to cause an effect upon lncRNA-CTS function, thus implying that there is a key relationship between lncRNA-CTS and the TGF-β/SMAD signaling pathway.

TGF-β receives substantial attention as a master inducer of EMT, as it participates in all types of EMT. TGF-β1 acts via both SMAD-dependent and SMAD-independent pathways which overlap and function collectively to regulate a range of transcription factors, including ZEB2 [28–30]. The TGF-β signaling cascade is started by specific binding between ligands and type II receptors. Activated type I receptors phosphorylate the intracellular effectors, SMAD2/SMAD3, which form complexes with SMAD4 and then translocate into the nucleus where these factors subsequently induce the transcription of target genes [31,32]. For example, the complex binds directly to regulatory promoter sequences of Snail, inducing its transcription, and subsequently, an active complex formed by SMAD3/SMAD4 and Snail can bind to regulatory promoter sequences of the genes encoding the epithelial junction proteins E-cadherin and occludin, leading to TGF-β1-induced repression of their expression. Our data showed that lncRNA-CTS can activate SMAD-dependent pathways by targeting miR-505, thereby promoting EMT in CC. Moreover, ZEB2 is known to play a role in the TGF-β/SMAD pathway by interacting with SMAD factors and acts as a multi-zinc finger protein that shows specific DNA binding activity and interacts with SMAD [33]. Collectively, these data suggest that ZEB2 is involved in activating the TGF-β/SMAD *via* lncRNA-CTS. Interestingly, we also found that lncRNA-CTS could regulate the expression of TGF-β receptor type II (TGF-βRII). TGF-βRII is a key regulator of the TGF-β/SMAD pathway. However, how lncRNA-CTS becomes integrated in the TGF-β/SMAD signaling pathway remains unknown and is presently under further investigation.

Although our research revealed that lncRNA-CTS can influence the process of EMT and metastasis in CC, we do not yet know whether lncRNA-CTS can affect proliferation and apoptosis in CC as this requires further research. It is well known that one lncRNA can function as a ceRNA for more than one form of miRNA, thereby exerting different biological functions. Whether lncRNA-CTS can influence the regulation of other miRNAs or proteins requires further research.

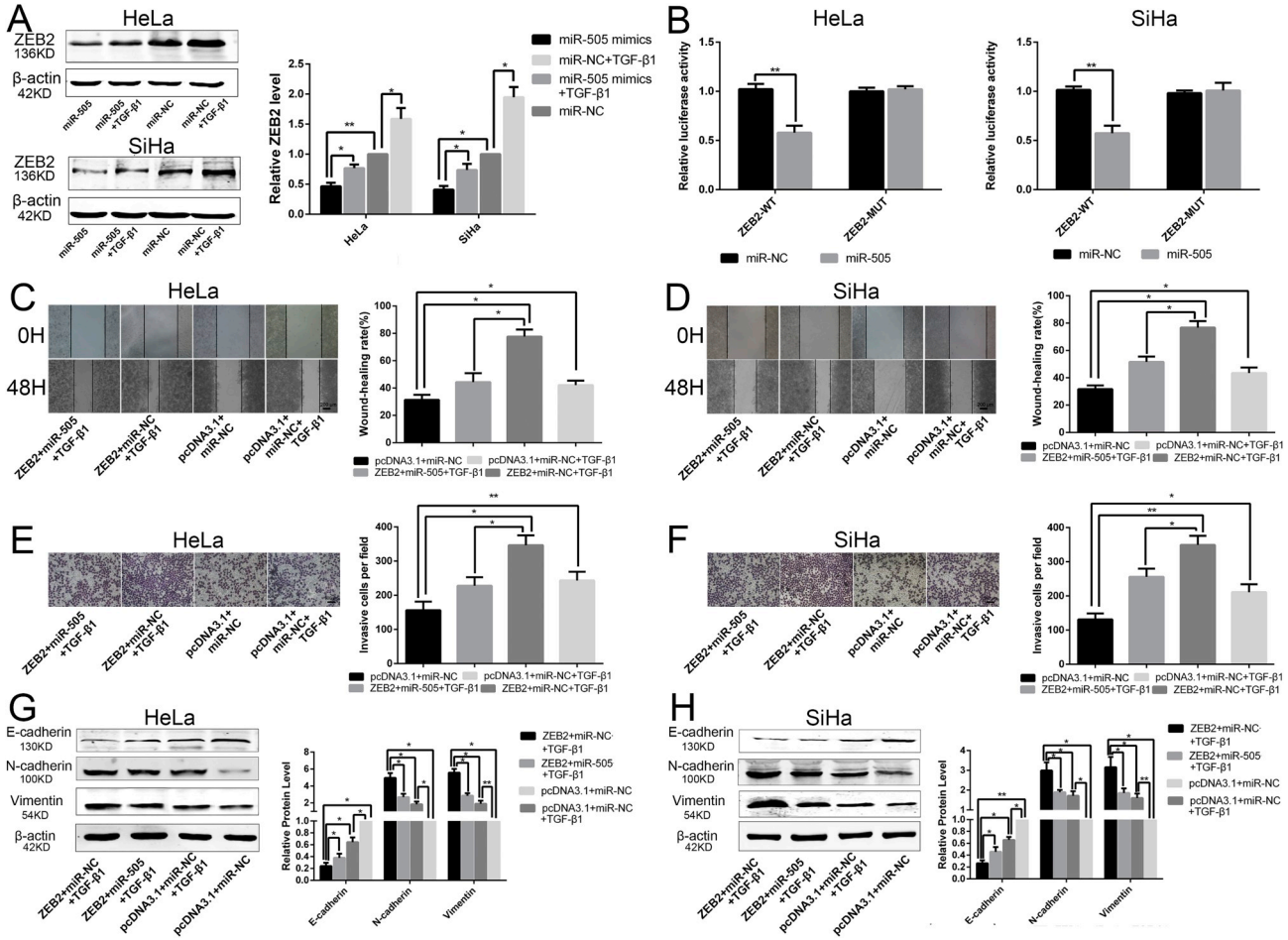


**Fig. 6.** miR-505 exerts influence over TGF-β1-mediated migration, invasion and EMT by targeting ZEB2. (**a)** Protein levels of ZEB2 expression in HeLa and SiHa cells transfected with miR-505 mimics, with or without TGF-β1, as determined by Western blotting assays. (**b**) The luciferase reporter activity of chimeric vectors carrying the luciferase gene and a fragment of ZEB2 containing the WT binding site or mutated binding site for miR-505. (**c, d)** Wound assays were carried out in both HeLa and SiHa cells which had been treated in specific ways. HeLa and SiHa cells were transfected with or without pcDNA3.1-ZEB2 and miR-505 mimics, in the presence or absence of TGF-β1. (**e, f)** Transwell assays were performed in HeLa and SiHa cells which had been treated as specified, after 48 h of incubation. (**g, h)** Protein levels of EMT markers in HeLa and SiHa cells which had been treated as specified, as determined by Western blotting assays. All data are presented as mean ± SD from at least three independent experiments. $*P < 0.05$, $**P < 0.01$.

## 5. Conclusions

In summary, our data show that the overexpression of lncRNA-CTS leads to a poor prognosis in CC patients, and that lncRNA-CTS promotes TGF-β1-induced EMT and metastasis by regulating the expression of ZEB2 via miR-505, both *in vitro* and *in vivo*. Furthermore, lncRNA-CTS was shown to activate the TGF-β/SMAD signaling pathway. Our data provide new insights into potential new mechanisms and strategies for therapeutic intervention of cancer metastasis.

## Ethical approval and consent to participate

All experiments were approved by the Ethics Review Committee of the Second Affiliated Hospital of Harbin Medical University.

## Consent for publication

All authors consented to the publication of this manuscript.

## Availability of supporting data

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Funding sources

This study was unfunded.

## Author contributions

Shujun Feng and Wenhua Tan designed the research. Shujun Feng, Wei Liu, and Zhaoyang Jia performed cellular experiments and animal experiments. Shunjin Zhang performed the bioinfomatics analysis. Wenjing Pan and Shujun Feng provided the samples and carried out

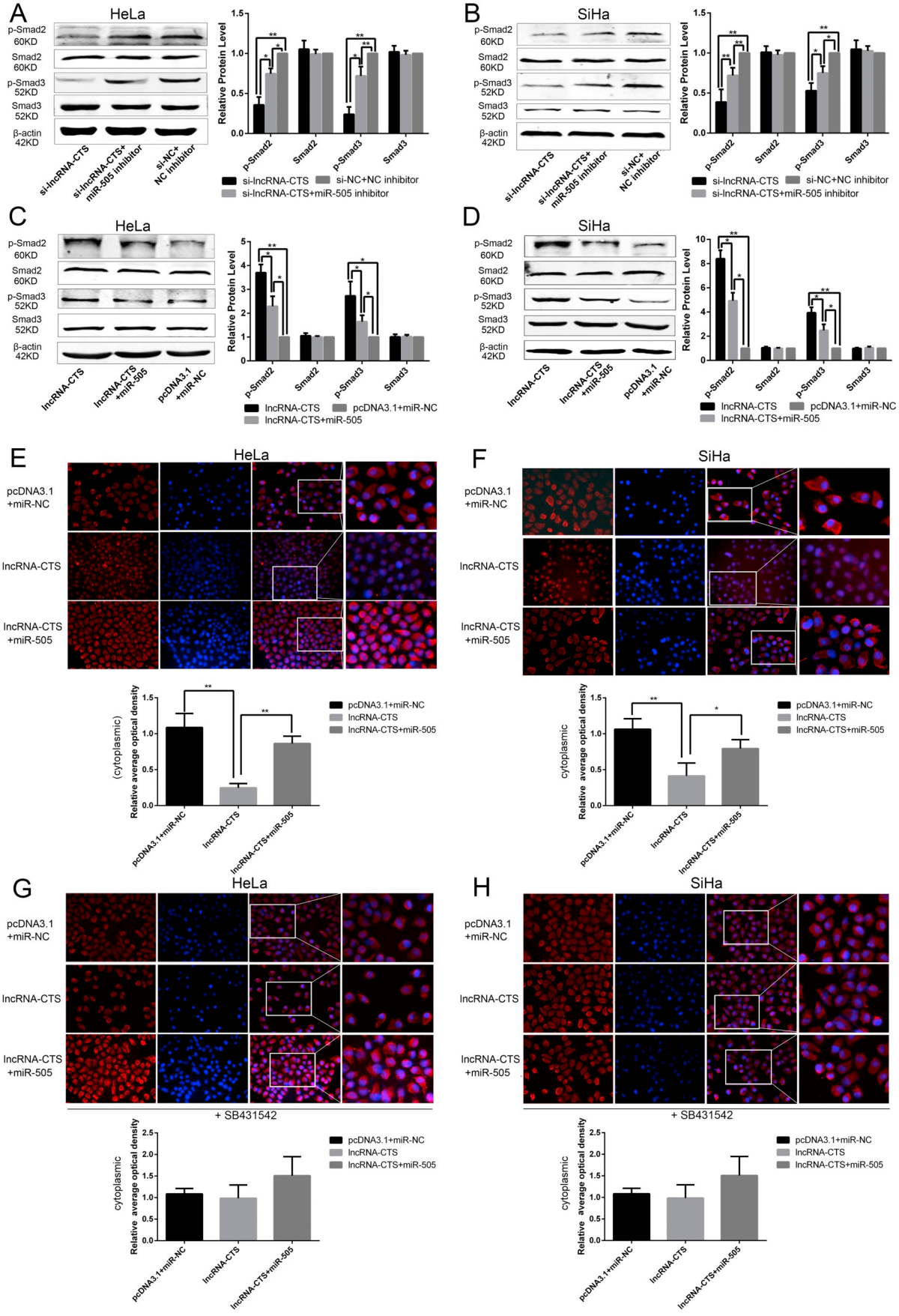


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**Fig. 7.** LncRNA-CTS and miR-505 were shown to regulate the TGF/SMAD signaling pathway. **(a, b)** Protein levels in HeLa and SiHa cells transfected with miR-505 inhibitor and si-lncRNA-CTS, as determined by Western blotting. **(c, d)** Protein levels in HeLa and SiHa cells transfected with pcDNA3.1-lncRNA-CTS and miR-505 mimics, as determined by Western blotting. **(e, f)** The distribution of SMAD2/3 was revealed by immunofluorescent assays in HeLa and SiHa cells transfected with pcDNA3.1-lncRNA-CTS and miR-505 mimics. **(g, h)** The distribution of SMAD2/3 was revealed by immunofluorescent assays in HeLa and SiHa cells transfected with pcDNA3.1-lncRNA-CTS and miR-505 mimics with SB431542 in HeLa and SiHa cells. All data are presented as mean ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

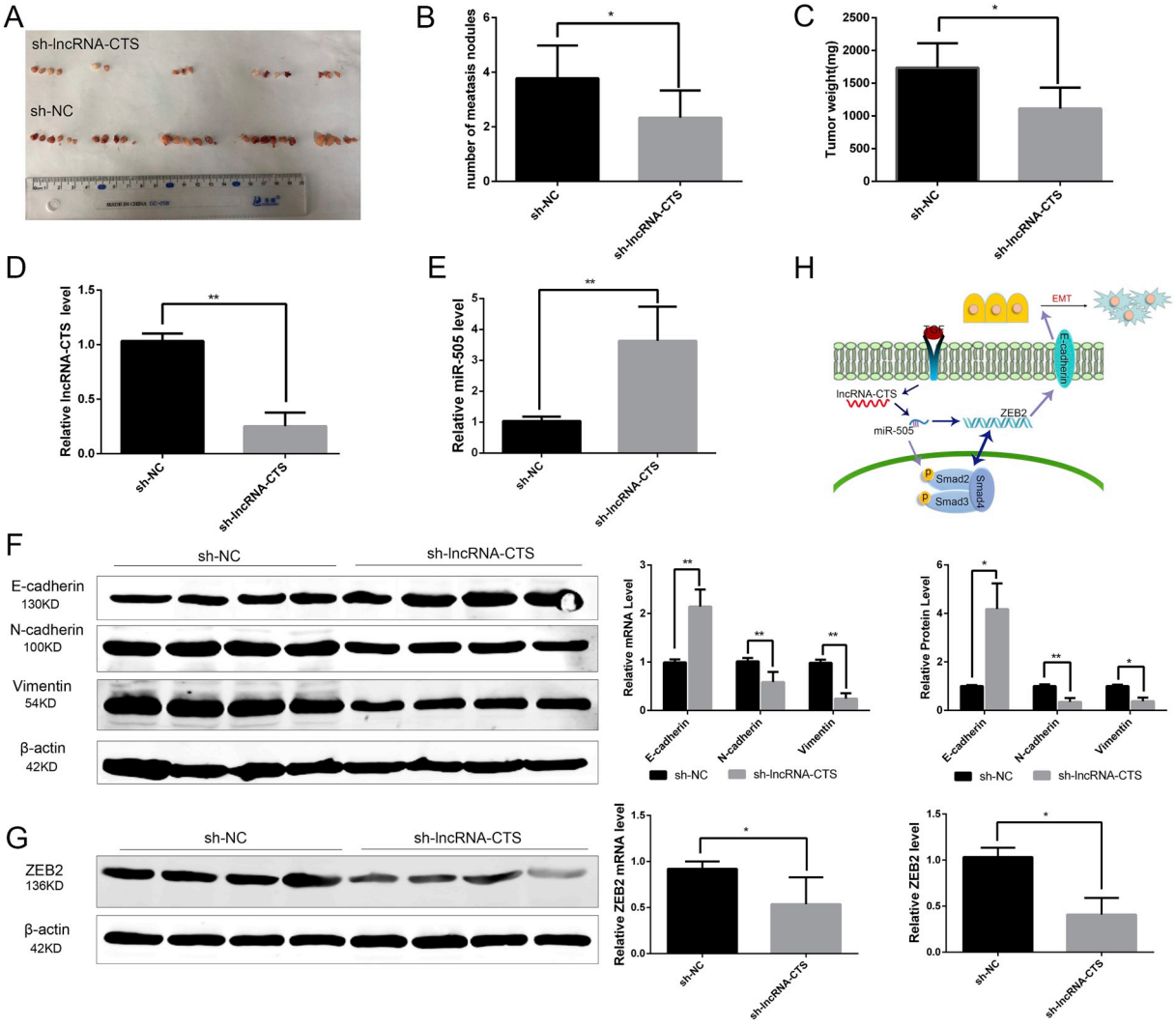


**Fig. 8.** The knockdown of lncRNA-CTS inhibited tumor progression *in vivo*. **(a, b)** Macroscopic observation of the size and number of metastatic nodules in nude mice. (n = 9) **(c)** Tumor weight after treatment with sh-lncRNA-CTS in nude mice. **(d)** Expression of lncRNA-CTS after treatment with sh-lncRNA-CTS in nude mice. **(e)** Expression of miR-505 after treatment with sh-lncRNA-CTS in nude mice. **(f)** The expression of epithelial-mesenchymal-transition (EMT) associated markers in tissues derived from xenograft tumors, as determined by qRT-PCR and Western blotting, respectively (n = 9). **(g)** Expression of ZEB2 in tissues derived from xenograft tumors, as determined by qRT-PCR and Western blotting, respectively (n = 9). **(h)** Potential schematic pathway illustrating the role of lncRNA-CTS in the process of EMT in CC. All data are presented as mean ± SD from at least three independent experiments.$^{*}P < 0.05$, $^{**}P < 0.01$.

pathological analysis. Shujun Feng and Xiaoxu Bai wrote the manuscript. All authors read and approved the final manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.09.002.

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