

Long non-coding RNA RP11-480I12.5 promotes cervical carcinoma progression by regulating the Wnt/ β -catenin signaling pathway

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Received June 7, 2019; Accepted October 10, 2019

DOI: 10.3892/ol.2019.11120

Abstract. The long non-coding RNA (lncRNA), RP11-480I12.5 is one of the most dysregulated lncRNAs, which is believed to contribute to the progression of cervical carcinoma (CC); however, the exact function of RP11-480I12.5 in human CC remains unknown. The present study aimed to investigate the function and underlying molecular mechanism of RP11-480I12.5 in CC. First, reverse transcription-quantitative PCR was implemented in order to detect differences in the expression of RP11-480I12.5 between normal and CC tissues. The present study used *in vitro* analysis to establish RP11-480I12.5 stable knockdown and overexpressing cell lines, in order to investigate the function and potential molecular mechanism of RP11-480I12.5 in the progression of CC. RP11-480I12.5 was upregulated in CC tissue compared with normal tissue. Furthermore, RP11-480I12.5 was associated with clinical stage, tumor size and lymph node metastasis. RP11-480I12.5 promoted the proliferation, migration and invasion of CC cell lines. Subsequently, the present study investigated the association between RP11-480I12.5 and the epithelial-to-mesenchymal transition (EMT) and Wnt/ β -catenin pathways. RP11-480I12.5 promoted EMT through the Wnt/ β -catenin pathway. Overall, the results of the present study demonstrate that RP11-480I12.5 promotes cervical cancer cell migration, invasion and EMT through the Wnt/ β -catenin pathway.

Introduction

Cervical cancer (CC) is one of the most common gynecological carcinomas worldwide with an incidence rate of ~3.2% in 2018 (1). Although advancements in radiotherapy and immunotherapy have been applied (2), ~50% of patients with late FIGO stage CC, a clinical stage system based on the location and metastasis of Tumor (3), remain to suffer from recurrence and metastasis. A previous study demonstrated that the overactivation of oncogene pathways such as Wnt/ β -catenin was associated with the progression of CC (4). A number of oncogenes have been reported to be engaged in the overactivation of the Wnt/ β -catenin pathway, including long non-coding RNA (lncRNA) CALML3-AS1, which was demonstrated to be upregulated and promote cell proliferation and metastasis in CC (5). It is believed that inhibiting the Wnt/ β -catenin pathway could partially restore the biocharacter of the oncogene induced to CC (6). Furthermore, numerous biomarkers predicting the prognosis of CC exert their function through the Wnt/ β -catenin pathway (7). However, the present study intended to establish novel biomarkers predicting prognosis and therapeutic targets for clinical application.

lncRNAs are members of non-coding RNAs (ncRNAs) that participate in a number of physiological and pathological processes, including cell division and differentiation (8). lncRNAs have been reported to be involved in the progression of several different types of carcinoma, including gastric, breast and lung cancer (9,10). Several studies have demonstrated that lncRNAs contribute to multiple-processes involved in CC, such as cell proliferation, angiogenesis and tissue invasion (11-13).

The present study scanned The Cancer Genome Atlas (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) database and identified RP11-480I12.5 as significantly upregulated in CC. The differences in the expression of RP11-480I12.5 between normal and CC tissues were detected. Subsequently, the biological function of RP11-480I12.5 was determined through a series of experiments and it was demonstrated that RP11-480I12.5 promotes the cervical cancer cell proliferation, migration and invasion of CC through the epithelial-to-mesenchymal transition (EMT) and Wnt/ β -catenin signaling pathways.

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Key words: cervical cancer, long non-coding RNA, migration and invasion, epithelial-to-mesenchymal transition

Materials and methods

Tissue samples. All human tissues were obtained from the Department of Gynaecology and Obstetrics, Jinan Women and Children Health Hospital (Jinan, China) after confirmation by a pathologist from July 2014 to January 2015. The inclusion criteria was as follows: Diagnosed as CC by pathologists between July 2014 and January 2015 in the Department of Gynaecology and Obstetrics, Jinan Women and Children Health Hospital. Patients with metastasis were excluded. A total of 50 patients were enrolled, (range, 45-71 years) and the mean age was 60.1±3.5 years old. A total of 50 cases were divided into four groups, according to their clinical features and the TNM stage system (14): Stage I, 7 cases; stage II, 19 cases; stage III, 9 cases and stage IV, 15 cases. Non-cancerous tissues were taken >5 cm from the tumor site and confirmed by a pathologist as normal tissue. All patients provided written informed consent and the present study was approved by the institution's Institutional Review Board.

Cell culture. The normal cervical epithelial ECT cell line PCS-480-011, and the human CC cell lines were obtained from the American Type Culture Collection. SiHa (HTB-35), HeLa229 (CCL-2.1) and MS751 (HTB-34) cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin (PS), whereas the C33A (HTB-31), HeLa (CCL-2) and Caski (CRM-CRL-1550) cell lines were cultured in DMEM F-12 medium (cat. no., 11330057; Thermo Fisher Scientific, Inc.) containing 10% FBS, at 37°C under 5% CO₂. SiHa cells are squamous cell (epidermoid) carcinoma grade II; HeLa 229, adenocarcinoma; MS751, epidermoid carcinoma and ECT cells are normal epithelium cells.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cancer cell lines and cancer tissues and normal tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed using the SYBRGreen detection RT-PCR system (Takara Bio, Inc.) with RT mix (cat. no., RR036B; Takara Bio, Inc.) and SYBRGreen (cat. no., 740703; Takara Bio, Inc.). Thermocycling conditions were as follows: Initial denaturation: 95°C for 5 sec; 40 cycles, 95°C for 5 sec of denaturation, 95°C for 35 sec and 60°C for 30 sec for annealing and elongation and 60°C for 30 sec for final extension. Actin was applied for normalization. The following primer sequences were used for the qPCR: E-cadherin: Forward, 5'-AACTCCACCTCCTGAAGCTG-3' and reverse, 5'-TTGCTTGACCTACTGCCAGA-3'; N-cadherin: Forward, 5'-TCCACCTACCTCCTGAAGCTG-3' and reverse, 5'-TTGACCACAGCTGTGAC-3'; Snail: Forward, 5'-ACCAAC TACCAACACCAAG-3' and reverse, 5'-TACCCACCAAGC TGTAG-3'; Slug: Forward, 5'-GGTATCATGGTCGGTATG GGT-3' and reverse, 5'-TCTTTACGACAGTGGTGGAGA-3'; Vimentin: Forward, 5'-TCCTACGTTGGTGTGAAGCT-3' and reverse, 5'-TTCTCTTTTACGACAGTGGTGG-3'; Actin: Forward, 5'-TCATGGTCGGTATGGGTCAA-3' and reverse, 5'-TCAGCAGTGGTGGAGAAAGA-3'; and RP11-480I12.5: Forward, 5'-ACGTTGGTGTGATGAAGCTCAA-3' and reverse,

5'-GCAGTGGTGGAGAAAGAGTA-3'. All experiments were performed in triplicate. The 2^{-ΔΔC_q} method was used to calculate relative RNA expression (15-17).

Colony formation assay. Transfected CC cells were seeded in 6-well plates at a density of 500 cells/well. Following a 2-week culture period, cell colonies were fixed for 20 min with 4% paraformaldehyde and stained with 1% crystal violet at room temperature. The colonies were examined and counted under a light microscope (Olympus FV100; magnification, x4). The colony index was the ratio of the colony number and the cells seeded onto the plates.

Transwell and Matrigel assays. Matrigel chambers were used in order to determine the invasion ability of the cervical cancer cells described above, according to the manufacturer's protocol (<https://www.thermofisher.com/order/catalog/product/140644?SID=srch-srp-140644>). A total of 5×10⁴ cells/well were resuspended in 200 μl plain medium (DMEM or DMEM F-12 described above) with no FBS in the upper chamber of a Transwell system, whereas the lower chamber was filled with 0.5 ml of medium supplemented with 10% FBS. BML284 (5 μg/ml) was added to the complete medium when necessary. Following incubation at 37°C for 24 h, the invasive cells were fixed with 100% methanol at room temperature for 20 min and stained with 0.5% crystal violet for 10 min. The migratory cells in the lower chamber were observed using an inverted microscope (magnification, x200). For the migration assay, the indicated cells (5×10⁴) were plated onto uncoated upper chambers. Cells were fixed with 100% methanol at room temperature and stained with 0.5% crystal violet. The number of transmembrane cells was observed under a light microscope (Nikon Corporation) at x200 magnification.

Wound healing assay. A total of 1×10⁵ cells/well were seeded in 6-well plates. Cells were incubated with medium (1% FBS and 1% PS with the corresponding medium as aforementioned) at 37°C for 48 h. Cells were not scratched and were not cultured in gapped inserts with barriers. Once the cells had reached 100% confluency, equal wounds were made with the 1 ml-pipette and the medium was replaced with total plain medium with no FBS. Following incubation for 24 h, the relative width was measured by the ratio of area and length. The area was calculated through ImageJ 1.49 (National Institutes of Health), according to the manufacturer's protocol, images were obtained with light microscope Olympus FV100 at 200x magnification.

Lentiviral production and stable cell line construction. Lentiviral vectors expressing a short hairpin RNA (shRNA, shRNA-1, shRNA-2 4 μg), empty vector (4 μg) as control and RP11-480I12.5 (4 μg) were co-transfected with the packaging vectors, psPAX2 (1 μg) and pMD2G (3 μg) (Addgene) into 293FT cells (cat. no., ACS-4500; ATCC) for lentiviral production using Lipofectamine[®] 3,000, according to the manufacturer's protocol. In order to establish stable cell lines, cells were transduced by using the aforementioned lentiviruses with polybrene (8 mg/ml, Sigma-Aldrich; Merck KGaA). HeLa cell line was infected with the shRNA lentiviruses to knock down HeLa, while C33A was infected with overexpressed lentivirus and control lentivirus. Following incubation for 72 h

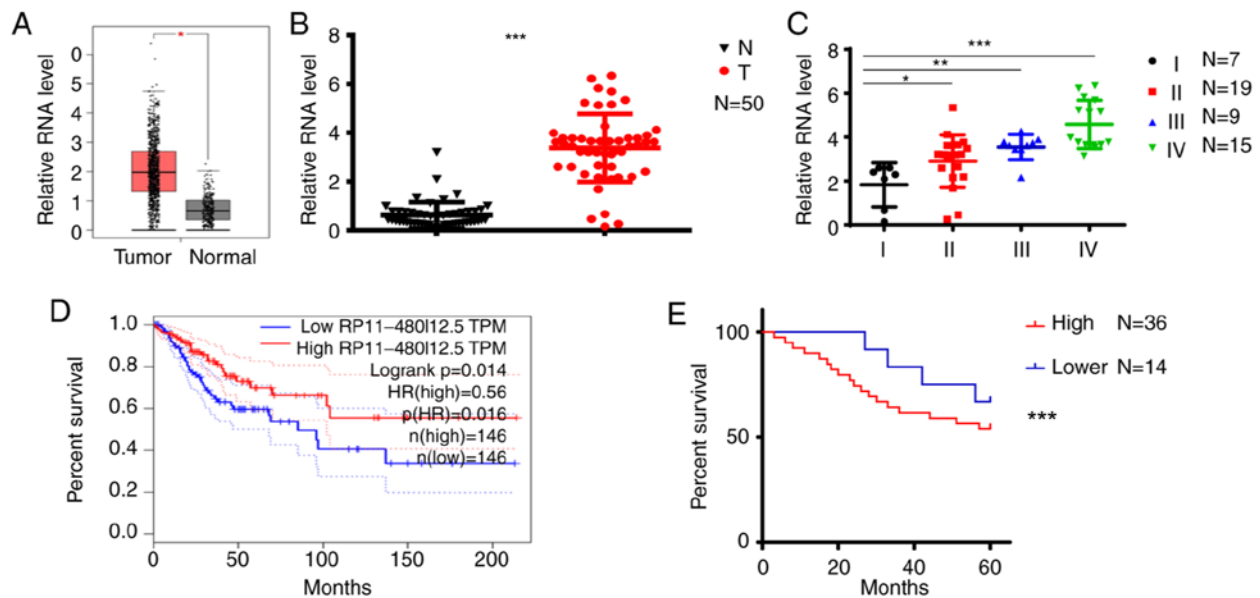


Figure 1. RP11-480I12.5 is upregulated in CC tissues and negatively associated with prognosis. (A) Relative level of RP11-480I12.5 in TCGA database. (B) Relative level of RP11-480I12.5 in the in-house database containing 50 cases (two-tailed paired Student's t-test, (***) $P<0.001$). (C) Relative level of RP11-480I12.5 at different stages of CC. One-way ANOVA test followed by Tukey's post hoc test, (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). (D) Survival analysis in TCGA database. (E) Survival analysis in the in-house database containing 50 cases. CC, cervical cancer; TCGA, The Cancer Genome Atlas; N, non-cancerous; T, tumor; TPM, Transcripts PerKilobase Million; HR, hazard ratio.

at 37°C, cervical cancer cells described above were supplemented with 2 mg/ml puromycin for 3 days. The details of the shRNA and plasmids are presented in Table SI.

CCK-8 assay. Stable CC cells were seeded in 96-well plates at a density of 200 cells/well. Following cell culture with complete medium (10% FBS added) for 24, 48 and 72 h, respectively, CCK-8 (Beyotime Institute of Biotechnology) was added and incubated for 2 h at 37°C. The absorbance was measured at 450 nm and the viability was normalized to the 0 h.

Edu assay. Transfected CC cells were seeded in 96-well plates at a density of 500 cells/well. Following incubation for 24 h at 37°C, Edu was added according to the manufacturer's protocol (RayBiotech). Five fields of view were randomly selected and images were captured using an Olympus FV100 microscope with red light fluorescence (magnification, x200). The percentage of Edu⁺ cells was calculated by the confocal automatically.

Western blotting. The cells (HeLa-NC, HeLa-sh, HeLa-sh2, C33A-NC and C33A-OV) were collected and lysed with RIPA buffer (Beyotime Institute of Biotechnology) and cell protein was obtained. The protein was quantified using the BCA kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Proteins (20 µg/lane) were separated via SDS-PAGE (12% gel). The protein was transferred onto a polyvinylidene fluoride membrane and blocked with 5% milk for 1 h at room temperature. The membranes were labeled with the corresponding primary antibodies; N-cad (cat. no. 13116), E-cad (cat. no. 14472), Vimentin (cat. no. 5741), Snail (cat. no. 3879), Slug (cat. no. 9585), β-catenin (cat. no. 8480), PCNA (cat. no., 13110), MMP7 (cat. no., 3807), MMP9 (cat. no., 13667) and β-actin (cat. no. 3700) (all 1:1,000; all from

Cell Signaling Technology, Inc.), and incubated overnight at 4°C. Following the primary incubation, membranes were incubated with the goat anti-rabbit and goat anti-mouse secondary antibody (1:10,000; cat. no. 5724, KPL, Inc.) for 1 h at room temperature. Protein bands were visualized using ECL (cat. no., 32209; Thermo Fisher Scientific, Inc.), the relative level of protein was normalized to β-actin.

TCGA database analysis. The datasets of dysregulated genes of cervical cancers was downloaded from TCGA database, the cervical carcinoma dataset (18), and a total of 306 cancers and 13 normal tissues were enrolled. The relative expression was analyzed by two-tailed paired Student's t-test.

Statistical analysis. Statistical analyses were performed using SPSS software (version 18; SPSS, Inc.) Data are presented as the mean ± standard deviation. All experiments were repeated in triplicate. The χ^2 test was used to analyze clinicopathological characteristics. The survival Kaplan-Meier analysis was applied by log-rank analysis. For patients enrolled, RP11-480I12.5 high was defined as those exhibiting higher than the average RNA level of the whole cohort of all the patients, while the remaining were defined as RP11-480I12.5 low. Comparisons between the two groups (normal tissue and cancer tissue) were evaluated using two-tailed paired Student's t-test and a similar variance was confirmed. One-way ANOVA was applied when making comparisons in datasets, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RP11-480I12.5 is upregulated in CC tissues and associated with a poor prognosis. The present study scanned

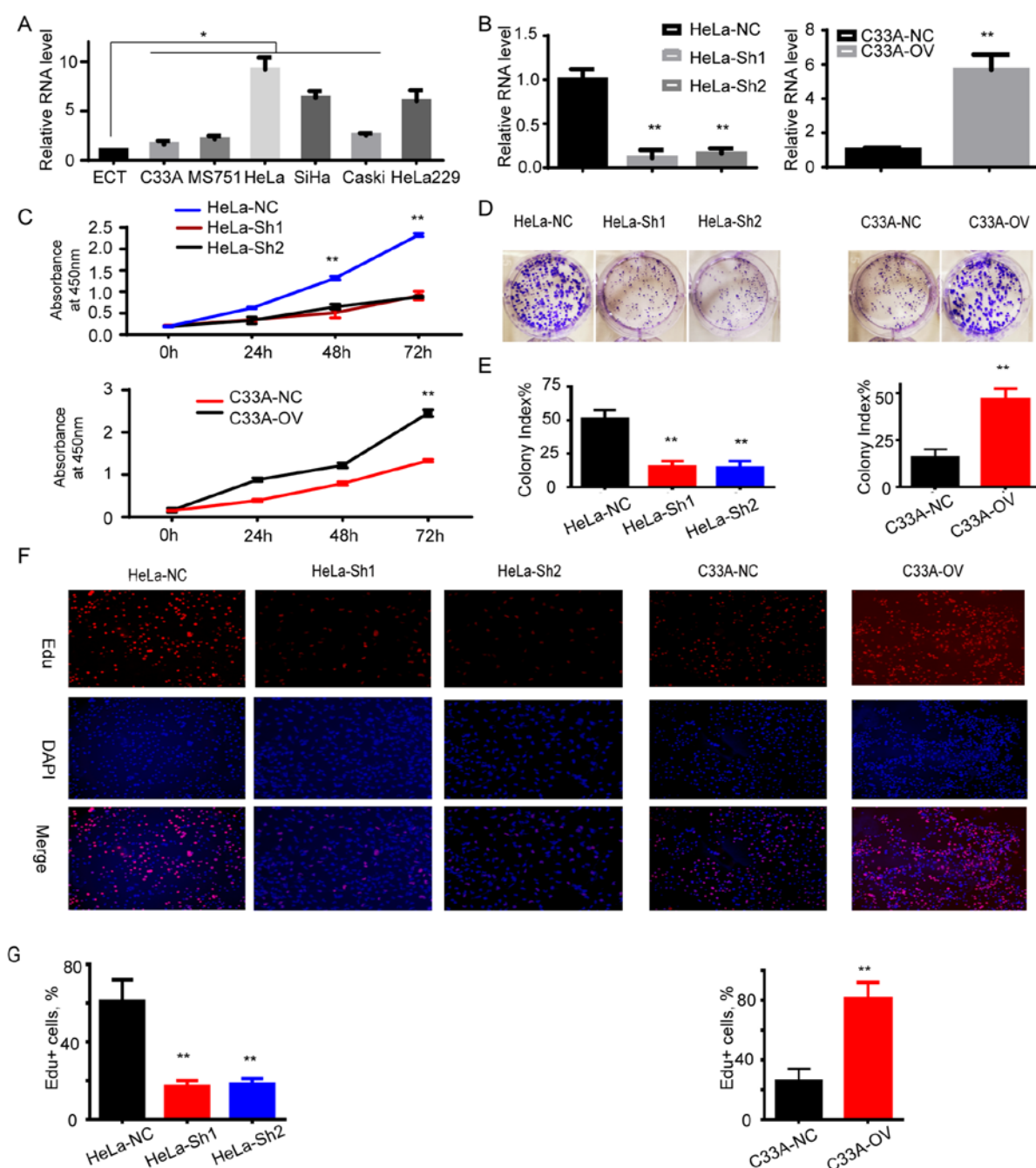


Figure 2. RP11-480I12.5 promotes the proliferation of CC. (A) Relative level of RP11-480I12.5 in the CC and normal cell lines. (B) Relative level of RP11-480I12.5 in cell lines. (C) Absorbance at 450 nm in the different cell lines. (D) Representative image from the colony formation assay. (E) Colony index of the indicated cells. (F) Representative image of Edu in different cell lines (magnification, $\times 200$). (G) Statistical analysis of Edu⁺ cell lines. One-way ANOVA test followed by Tukey's post hoc test, * $P < 0.05$; ** $P < 0.01$ vs. NC. CC, cervical cancer; sh, short hairpin; OV, overexpression; NC, negative control.

TCGA database and identified RP11-480I12.5 as one of the lncRNAs contributing to the progression of CC (Fig. 1A). To further verify this hypothesis, the present study analyzed the expression differences in 50 paired normal and tumor tissues. RP11-480I12.5 was significantly upregulated in CC tissues compared with normal tissues ($P < 0.001$; Fig. 1B). Subsequently, the level of RP11-480I12.5 was significantly higher in the later stages of CC compared with stage I tumors ($P < 0.001$; Fig. 1C). Furthermore, the present study analyzed the association between RP11-480I12.5 and the overall survival of patients. Both TCGA database ($P < 0.001$; Fig. 1D) and the in-house database containing the aforemen-

tioned 50 cases used in the present study ($P < 0.001$; Fig. 1E) demonstrated that RP11-480I12.5 was negatively associated with prognosis.

RP11-480I12.5 promotes the proliferation of CC. The present study demonstrated that RP11-480I12.5 is upregulated in CC and negatively associated with prognosis. In order to further determine the biological function of RP11-480I12.5, the present study examined its expression pattern in HeLa and C33A CC cell lines described above. The CC cell line was demonstrated to express a significantly higher level of RP11-480I12.5 compared with the normal control tissue,

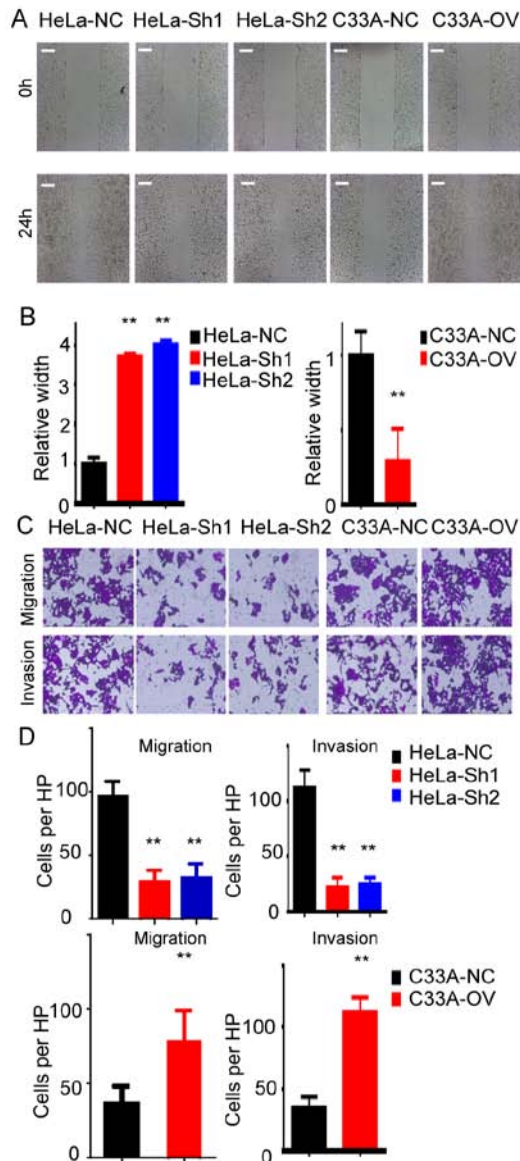


Figure 3. RP11-480I12.5 promotes the migration and invasion of CC. (A) Representative images of wound healing assay (Scale bar, 50 μ m). (B) Statistical analysis of wound healing. (C) Representative image of the Transwell and invasion chamber assays. (D) Statistical analysis of the Transwell and invasion chamber assays. One-way ANOVA test followed by Tukey's post hoc test, ** $P < 0.01$ vs. NC. CC, cervical cancer; NC, negative control; sh, short hairpin; OV, overexpression.

ECT ($P < 0.05$; Fig. 2A). Subsequently, the present study established the RP11-480I12.5 stable knockdown cell line in HeLa cells and the RP11-480I12.5 overexpression cell line in C33A cells. The relative RNA level is presented in Fig. 2B ($P < 0.001$), which indicated that it increased in RP11-480I12.5 overexpression cell lines, while decreased in knocked down cell lines, indicating that stable cell lines were established. Those cells with higher levels of RP11-480I12.5 demonstrated a higher absorbance at 450 nm ($P < 0.001$; Fig. 2C), a higher colony index ($P < 0.001$; Fig. 2D and E) and a higher percentage of Edu⁺ cells ($P < 0.001$; Fig. 2F and G). The results of the present study indicate that RP11-480I12.5 promotes cellular proliferation of CC.

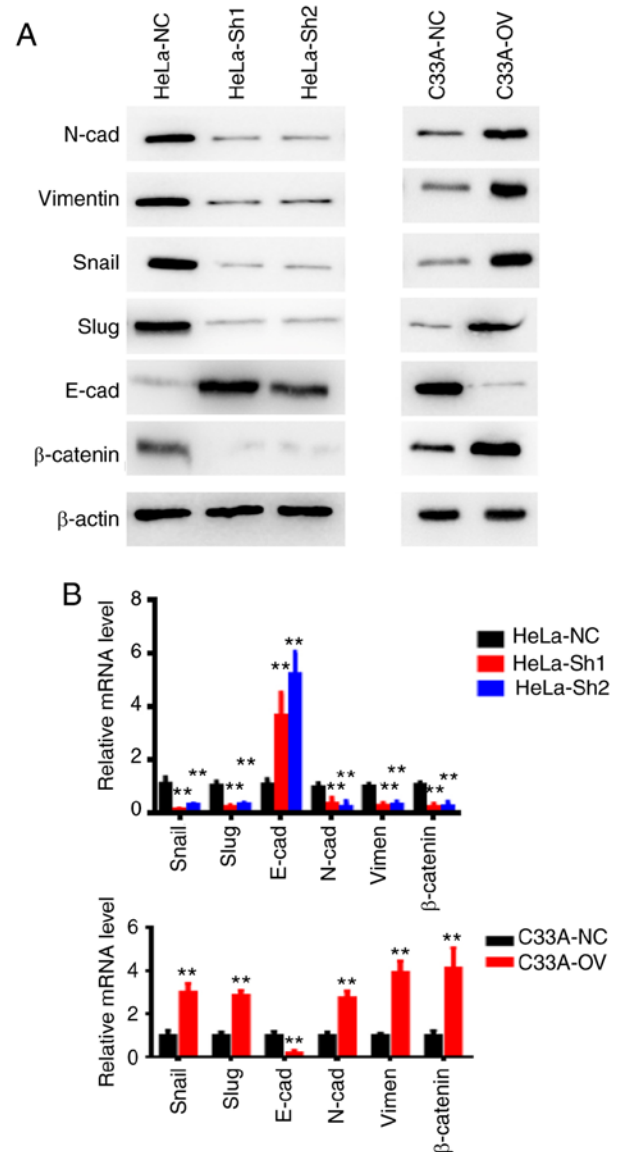


Figure 4. RP11-480I12.5 induces EMT in CC through the Wnt/ β -catenin pathway. (A) Western blot analysis of EMT markers and β -catenin. (B) Relative RNA levels of EMT markers and β -catenin. One-way ANOVA test followed by Tukey's post hoc test, ** $P < 0.01$ vs. NC. EMT; epithelial-to-mesenchymal transition; CC, cervical cancer; NC, negative control; sh, short hairpin; OV, overexpression.

RP11-480I12.5 promotes the migration and invasion of CC. Recurrence and metastasis are the most common reasons contributing to the mortality of patients with cancer (19). The present study analyzed the migration and invasion abilities of the indicated cell lines. The results of the present study demonstrated that cells with higher levels of RP11-480I12.5 indicated an improved migration ability. The cells harbored improved migration ability in the wounds healing assay in HeLa-NC and C33A-OV cells compared with the control cells ($P < 0.01$; Fig. 3A and B). The Transwell and matrigel assays demonstrated that cells with higher levels of RP11-480I12.5 had enhanced migration and invasion abilities ($P < 0.01$; Fig. 3C and D).

RP11-480I12.5 induces EMT by promoting the Wnt/ β -catenin pathway. The present study previously demonstrated that RP11-480I12.5 promotes cell proliferation, migration and

invasion. To further investigate the potential underlying molecular mechanism involved, the present study examined EMT markers and β -catenin in the indicated cells. The mesenchymal markers, including N-cadherin, Vimentin, Snail and Slug were decreased in cervical cancer cells with higher levels of RP11-480I12.5, while the epithelial markers such as E-cadherin were increased, as expected. These results are consistent with the aforementioned proliferation and migration and invasion functions. The relative mRNA levels of the EMT markers in the HeLa and C33A cell lines are presented in Fig. 4. The corresponding changes demonstrated in the proliferation marker and the matrix metalloproteinase protein are presented in Fig. S1. The Wnt pathway activator, BML284 was subsequently added to the C33A cell lines. The results of the present study demonstrated that RP11-480I12.5 activates the Wnt/ β -catenin pathway in a synergistic manner with BML 284 (Fig. S2), this may be due to the fact that they exert their function in different ways. Overall, the results of the present study indicate that RP11-480I12.5 induces the EMT of CC through the Wnt/ β -catenin pathway.

Discussion

Human CC is one of the most common malignancies worldwide. Patients with late-stage CC still suffer from recurrence and metastasis; however, the exact molecular mechanisms of tumorigenesis and progression remain unknown (19). Therefore, further studies are required in order to determine additional biomarkers and therapeutic targets.

LncRNAs are ncRNAs that play an important role in a number of physiological and pathological processes, including cell differentiation and division (20,21). An increasing number of studies have demonstrated that lncRNAs are involved in the tumorigenesis and progression of multiple types of cancer, including gastric and colon cancer (22,23). LncRNAs are known to exert their function on CC via multiple steps (24). For example, the lncRNA LOC105374902 promotes the malignancy of CC cells by acting as a sponge of miR-1285-3p, a process enhanced by TNF- α (25). Furthermore, the lncRNA SBF2-AS1 promotes the progression of CC by regulating the miR-361-5p/FOXM1 axis (26), while the lncRNA BDNF-Anti-Sense is downregulated in CC and possesses anti-tumor functions by negatively associating with brain-derived neurotrophic factor (27). RP11-480I12.5 is a newly identified lncRNA and, to the best of our knowledge, has not yet been extensively studied. The potential function and molecular mechanism underlying RP11-480I12.5 in CC remain unknown. Thus, the present study examined the level of RP11-480I12.5 in CC and its association with prognosis. Patients with higher levels of RP11-480I12.5 experienced shorter overall survival and disease-free survival. In order to determine the biological function of RP11-480I12.5, the present study detected the RNA level of RP11-480I12.5 in the CC cell line and established stable knockdown and overexpression cell lines in HeLa and C33A cells. Cells with higher levels of RP11-480I12.5 indicated improved proliferation, migration and invasion ability than cells with lower levels of RP11-480I12.5. The present study subsequently detected mesenchymal and epithelial markers in the indicated cells. RP11-480I12.5 was demonstrated to promote the transition from an epithelial status to a mesenchymal status.

Overall, the results of the present study demonstrate that RP11-480I12.5 promotes the cell proliferation, migration and invasion ability of human CC by inducing EMT through the Wnt/ β -catenin pathway.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

LS designed the present study. LS, YL and LZ were engaged into the performance of the research, analysis of the data and editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Jinan Women and Children Health Hospital Institutional Review Board. The Bioethics Commission number is 140707-003. Written consent was obtained from all patients prior to tissue collection. The Bioethics Commission number was 140707-003.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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