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LncSUMO1P3 exacerbates malignant behaviors of esophageal squamous cell carcinoma cells via miR-486-5p/PHF8/CD151

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ABSTRACT

Background: Esophageal squamous cell carcinoma (ESCC) is a malignancy usually associated with smoking or alcohol consumption. The involvement of long noncoding RNAs (lncRNAs) in the regulation of tumor development and metastasis through molecular mechanisms has been unveiled by accumulating evidence. However, the function of lncRNA SUMO1 Pseudogene 3 (lncSUMO1P3) essential to ESCC development remains obscure.

Methods: Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot (WB) analysis were done to measure RNA and protein levels. Functional assays were carried out to examine the changes in ESCC cell phenotype. Supported by bioinformatics analysis, mechanism assays were done for assessment of putative interactions among different genes.

Results: LlncSUMO1P3 was aberrantly up-regulated in ESCC cell lines, and lncSUMO1P3 deficiency could hamper cell proliferation, migration and invasion as well as epithelial-mesenchymaltransition (EMT) in ESCC while lncSUMO1P3 overexpression led to the opposite consequences. LncSUMO1P3 could competitively bind to microRNA-486-5p (miR-486-5p) or PHD finger protein 8 (PHF8) to modulate CD151 expression. CD151 was also verified to regulate ESCC cell biological behaviors.

Conclusion: Our study revealed that lncSUMO1P3, up-regulated in ESCC cells, could sponge miR-486-5p and recruit PHF8 to up-regulate CD151, thus influencing the malignant behaviors of ESCC cells.

1. Background

Esophageal squamous cell carcinoma (ESCC) is a subtype of esophageal cancer, severely affecting human health worldwide in recent years for its increasing incidence rate. Risk factors of ESCC include familial inheritance, gene mutation, excessive drinking or smoking, to name just a few [1]. As a health threat worldwide, the malignancy has been reported to be overwhelmingly more prevalent in Asia than in other regions [2]. Usually, ESCC patients are diagnosed in advanced stage for lack of specific symptoms presented in the early stage, and systemic treatment of advanced ESCC often includes cytotoxic agents, molecular-targeting agents and no molecular-targeting agents specifically for Phase 3 [3,4].

Long non-coding RNAs (lncRNAs) have been widely elucidated to perform critical roles in different biological processes and the dysregulation of lncRNAs has also been closely associated with development and metastasis of varied malignancies including ESCC.

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LncRNA small nucleolar RNA host gene 16 (SNHG16) was proved to exert promoting influences on cell invasive ability by regulating Wnt/β-catenin signaling pathway in ESCC [5]. LncRNA activated by TGF-Beta (lnc-ATB) was suggested as an oncogenic factor in ESCC via modulation on Kindlin-2 【28640252】. Moreover, lncRNA extracellular matrix (ECM), overexpressed in ESCC, could accelerate ESCC metastasis and might serve as a promising biomarker for ESCC diagnosis 【30128011】. However, lncRNA SUMO1 Pseudogene 3 (lncSUMO1P3), whose oncogenic role has already been discovered in various cancers including glioma and gastric cancer 【33179980, 33015800】, has never been studied in ESCC yet.

Reviewing previous studies, lncRNAs can regulate downstream gene expression by competing with mRNAs through microRNAs response elements (MREs) in miRNAs to bind miRNAs. ceRNAs can act as miRNA sponges and functionally prevent these targeted transcriptional mRNAs from being degraded by miRNAs【29115513】, lncRNAs can sponge microRNA (miRNA) to function as a competing endogenous RNA (ceRNA) and affect the expression of downstream genes 【31717266】 [6], which makes them natural miRNA sponges that function as ceRNAs and become indispensable in the anti-tumor or tumor-promoting process (Fig. S1). The ceRNA role of lncRNAs has been explored widely reported in ESCC. Specifically, lncRNA ZNFX1 Antisense RNA 1 (ZFAS1) has been discovered to bind with miR-124 to elevate expression of signal transducer and activator of transcription 3 (STAT3), thus exacerbating ESCC cell malignant behaviors [7]. Prostate cancer associated transcript-1 (PCAT-1) could function as miR-508-3p sponge to affect expression of Annexin A10 (ANXA10), thus contributing to accelerated ESCC progression [8]. Moreover, lncRNA prostate androgen regulated transcript 1 (PART1) was claimed to suppress ESCC cell proliferation and invasion via miR-18a-5p/SRY-box transcription factor 6 (SOX6) axis 【33432363】. Nevertheless, there have been no reports uncovering the ceRNA role of lncSUMO1P3 in ESCC by now.

In present research, the main aim was to fathom out lncSUMO1P3 role and the underlying molecular mechanism in ESCC cells by conducting a series of experiments, intending to provide a novel sight for understanding ESCC.

2. Methods

2.1. Cell culture

The ESCC cell lines EC109 was obtained from Cell Bank of Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. KYSE-450 (C1318, WHELAB, Shanghai, China) and KYSE-150 (C1015, WHELAB) and human normal epithelial squamous cell line Het-1A (CRL-2692, ATCC) were procured for this study. KYSE-450 cells were cultured in RPMI 1640 (M0200, WHELAB) +F12 (M0500, WHELAB) and other cell lines including EC109, KYSE-150, Het-1-A and HEK293T cells were cultivated in RPMI1640 Medium. The medium was supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin 100 × (PB180120, Procell) and 1% L-Glutamine. When the cell convergence reached 80%, the cells were treated with 0.25% trypsin (Vicmed, China) for sub-culturing in an incubator with 5% CO_2 at 37 °C. HEK293 cells provided by ATCC were cultured in DMEM High Glucose (Gibco) with 10% FBS and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37 °C. The characteristics of the cell lines used are shown in Table 1 below.

2.2. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted with Trizol reagent (Takara, Japan) on the basis of manufacturer's protocols. The extracted RNAs were reversely transcribed into cDNA synthesis with Reverse Transcriptase Kit (11141ES10, Takara). Then, qPCR analyses were carried out by utilization of ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control for lncRNAs and mRNAs while U6 for miRNAs. All results were calculated and presented as $2^{-\Delta\Delta Ct}$. The experiment was carried out with three biological replicates.

2.3. Plasmid transfection

Lentivirus-containing short hairpin RNAs (sh-RNAs) targeting lncSUMO1P3 and PHD finger protein 8 (PHF8), as well as the negative control (sh-NC) were procured from Nanjing BioChannel Biotechnology. Full length of lncSUMO1P3, PHF8 and CD151 was respectively sub-cloned into pcDNA3.1 vectors (45539, Biofeng) for overexpression of the indicated genes. Mimics NC, miR-486-5p mimics, inhibitor NC and miR-486-5p inhibitor were designed and procured from RiboBio. Lipofectamine 2000 reagent was utilized for plasmid transfection. Constructed plasmids were transfected into cells and stably transfected cells were selected after 48-h transfection. RT-qPCR was conducted to test the silence or overexpression efficiency. The experiment was carried out with three biological replicates.

Table 1
The characteristics of the cell lines used in our study.

Cell line	Name	Cell morphology	Culture
EC109	Human esophageal cancer cells	Adherent epithelial-like	RPMI1640 Medium
KYSE-450	Human esophageal squamous cells	Adherent monolayer epithelial cells	RPMI 1640 + F12
KYSE-150	Human esophageal squamous cells	Adherent monolayer epithelial cells	RPMI1640 Medium
Het-1A	Human normal epithelial squamous cell line	Adherent epithelial cells	RPMI1640 Medium
HEK293T	Human embryonic kidney cells	Adherent epithelial-like	RPMI1640 Medium

2.4. Cell counting kit-8 (CCK-8) assay

Cells stably transfected with target plasmids were seeded into 96-well plates and incubated with 5% CO_2 at 37 °C for 24 h. CCK-8 Kit (M4839, ABMOLE) was applied to test the cell viability according to the absorbance value at a wavelength of 450 nm. The experiment was carried out with three biological replicates.

2.5. Wound healing assay

Cells of different groups were seeded onto 6-well plates, and the plate back of each well was marked by at least 5 paralleled marker lines. When the cell confluence reaches 80%, each well was scratched vertically to the marker lines by sterile pipette. After scratching, wounded cells were washed away and fresh serum-free medium was replenished. Cells were incubated for 24 h with 5% CO_2 at 37 °C before being observed with microscope and taken pictures for analyzing. The experiment was carried out with three biological replicates.

2.6. Transwell invasion assay

Transwell filter chambers (BD Biosciences, New Jersey, USA) were utilized to test the invasive ability of cells. The apical chamber was supplied with serum-free medium while the basal chamber with 10% FBS (Gibco, USA) medium. Transfected cells were seeded into the apical chamber coated with Matrigel. After 24 h, the successfully invaded cells were fixed by methanol and dyed with Giemsa. Five fields were randomly chosen for cell counting under microscope. Image J was used for calculating number of successfully invaded cells. The experiment was carried out with three biological replicates.

2.7. Fluorescence in situ hybridization (FISH)

KYSE-450 and KYSE-150 cells grown on slides overnight were fixed with 4% paraformaldehyde for 15 min. The fix buffer was washed away and cells were treated with 0.5% Trition X-100 and Proteinase K. Then, the cells were cultured with lncSUMO1P3 FISH probes overnight. 4',6-diamidino-2-phenylindole (DAPI; R37606, Thermo Fisher) was utilized to counterstain nuclei. Zeiss LSM800 confocal microscopy was applied to observe the results. The experiment was carried out with three biological replicates.

2.8. RNA binding protein immunoprecipitation (RIP) assay

Imprint® RNA Immunoprecipitation Kit (RIP-12RNX, Sigma-Aldrich, USA) was utilized strictly according to user manual for RIP assay. Briefly, cell lysates were obtained by treating cells with lysis buffer and then incubated with bead-coated Anti-AGO2 (1/50), *Anti*-IgG (1/50) or *Anti*-PHF8 (1/200) at 4 °C overnight. Then, the precipitated RNAs were purified and analyzed by RT-qPCR. The experiment was carried out with three biological replicates.

2.9. RNA pulldown

RNA pulldown assay was performed with Pierce™ Streptavidin Magnetic Beads (88816, Thermo Fisher). 1 μL Bio-lncSUMO1P3, Bio-CD151 3′UTR or Bio-NC was treated by structure buffer and then cultivated with 15 μL streptavidin beads respectively. Then, the biotinylated RNA-bead complexes were co-cultured with cell lysates at 4 °C overnight. Subsequently, purified RNAs or proteins were analyzed through RT-qPCR or WB respectively. The experiment was carried out with three biological replicates.

2.10. Dual luciferase reporter assay

The full length of lncSUMO1P3 or fragment of CD151 3′UTR containing wide-type or mutated miR-486-5p binding sites was respectively sub-cloned into pmirGLO vectors (E1330, Biofeng) to construct pmirGLO-lncSUMO1P3-WT/Mut and pmirGLO-CD151 3′UTR-WT/Mut. The constructed plasmids were respectively co-transfected with mimics NC or miR-486-5p mimics into ESCC cells and HEK293T cells. Additionally, pGL3-CD151 promoter was obtained by sub-cloning the promoter sequence of CD151 into pGL3 vectors (E1761, Biofeng), which was then co-transfected with pcDNA3.1 or pcDNA3.1-PHF8 into ESCC cells and HEK293T cells. After co-transfection for 24 h, Dual Luciferase Reporter Gene Assay Kit (RG027, Beyotime) was applied to test the relative luciferase activity. The experiment was carried out with three biological replicates.

2.11. Western blot (WB)

Cells were treated with RIPA buffer (KeyGen Biotech Co., Ltd., Nanjing) and then total proteins were extracted with Total Protein Extraction Kit (PROTTOT-1KT, Sigma-Aldrich, USA). Proteins were separated with SDS-PAGE Gel Kit (P0670-250 ml, Beyotime Biotechnology, Shanghai) and then transferred to polyvinylidene fluoride (PVDF) membranes (Vicmed, China). Subsequently, the PVDF membranes were blocked with blocking buffer and incubated with Anti-CD151 antibody [11G5a] (1/2000; ab33315, abcam), *Anti*-PHF8 antibody, Recombinant *Anti*-E Cadherin antibody [EPR699] (1/10000; ab133597, abcam), *Anti*-N Cadherin antibody (1/10000; ab76057, abcam), *Anti*-Claudin-1 antibody (1/2000; ab15098, abcam), Recombinant *Anti*-Vimentin antibody [EPR3776] (1/

2000; ab92547, abcam), Recombinant Anti-SNAIL antibody [EPR21043] (1/1000; ab216347, abcam), or *Anti*-GAPDH antibody (1/1000; ab9485, abcam) respectively overnight at 4 °C. Then, the PVDF membranes were incubated with respective secondary antibodies for 1 h at room temperature. The protein was detected by BeyoECL Plus. The experiment was carried out with three biological replicates.

2.12. DNA pulldown

Biotin-labeled CD151 promoter or NC probes were co-cultured with cell lysates. Then, Pierce™ Streptavidin Magnetic Beads were utilized to pull down the biotin-labeled DNA-Protein duplexes. The proteins were eluted from DNA and detected through WB. The experiment was carried out with three biological replicates.

2.13. Statistical analysis

Statistical analyses in this study were conducted with the help of SPSS 22.0 Software. Student's t-test and analysis of variance (ANOVA) were performed to compare the mean values. Each experiment was performed in triplicate and all data were shown as the mean ± standard deviation (SD). $P < 0.05$ was considered to be statistically significant, and the techniques used are listed below (Table 2).

3. Results

3.1. LncSUMO1P3 was overexpressed in ESCC cells and exacerbated the malignant behaviors of ESCC cells

LncSUMO1P3 expression in ESCC cell lines and human normal epithelial squamous cell line (Het-1A) was first measured through RT-qPCR, and it was manifested that lncSUMO1P3 was highly expressed in ESCC cells (especially in KYSE-150 and KYSE-450 cells) compared with Het-1A (Fig. 1A). Subsequently, it was shown in RT-qPCR analysis that after respective transfection of sh-NC, sh-lncSUMO1P3 or pcDNA3.1, pcDNA3.1-lncSUMO1P3 into KYSE-150 and KYSE-450 cells, lncSUMO1P3 level was notably decreased by sh-lncSUMO1P3 and substantially increased by pcDNA3.1-lncSUMO1P3 (Fig. 1B–C). Subsequently, CCK-8 was carried out for assessment of lncSUMO1P3 influence on ESCC cell proliferation. As was revealed by the results, lncSUMO1P3 silence could weaken the proliferation ability of ESCC cells while lncSUMO1P3 overexpression caused totally opposite consequence (Fig. 1D–E). Based on wound healing assay results, cell migration was largely hampered by sh-lncSUMO1P3 but promoted by pcDNA3.1-lncSUMO1P3 (Fig. 1F–G). In addition, transwell assay was conducted to verify lncSUMO1P3 impacts on ESCC cell invasion. It was proved that cell invasive ability was also hindered by lncSUMO1P3 inhibition but improved by lncSUMO1P3 overexpression (Fig. 1H–I). Then, the transfected cells were observed under optical microscope. It could be found that ESCC cell EMT was lessened after transfection of sh-lncSUMO1P3 but was exacerbated after transfection of pcDNA3.1-lncSUMO1P3 (Fig. 1J). To sum up, lncSUMO1P3 was overexpressed in ESCC cells and could aggravate the malignant behaviors of ESCC cells.

3.2. LncSUMO1P3 could sponge miR-486-5p in ESCC cells

LncRNA is well-known to function as ceRNA at post-transcriptional phase to compete for binding to miRNAs [9]. Thus, we conducted FISH assay to locate lncSUMO1P3 in ESCC cells with LSM800 confocal microscopy. As expected, we found lncSUMO1P3 mostly existed in cytoplasm, which implied the possibility of ceRNA network (Fig. 2A). Subsequently, RIP assay results showed that lncSUMO1P3 could be largely precipitated in AGO2 antibody (Fig. 2B). Based on the fact that AGO2 could not bind with lncRNAs directly, it was reasonable to postulate that lncSUMO1P3 modulated the biological behaviors of ESCC cells through ceRNA network. We searched for potential downstream miRNAs of lncSUMO1P3 on LncBase Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) (Fig. 2C). Through RNA pulldown assay, among the 12 predicted miRNAs, only miR-486-5p was largely pulled down by Bio-lncSUMO1P3 (Fig. 2D). Additionally, the enrichment of lncSUMO1P3 was

Table 2
The techniques used in this study.

Name	Reagent Kits
RT-qPCR	Q711-02, Vazyme
Plasmid transfection	45539, Biofeng RiboBio
CCK-8 assay	M4839, ABMOLE
Wound healing assay	
Transwell invasion assay	BD Biosciences, New Jersey, USA
FISH	Thermo Fisher
RIP	RIP-12RNX, Sigma-Aldrich, USA
RNA pulldown	88816, Thermo Fisher
Dual luciferase reporter assay	RG027, Beyotime
Western blot	KeyGen Biotech Co., Ltd., Nanjing
DNA pulldown	

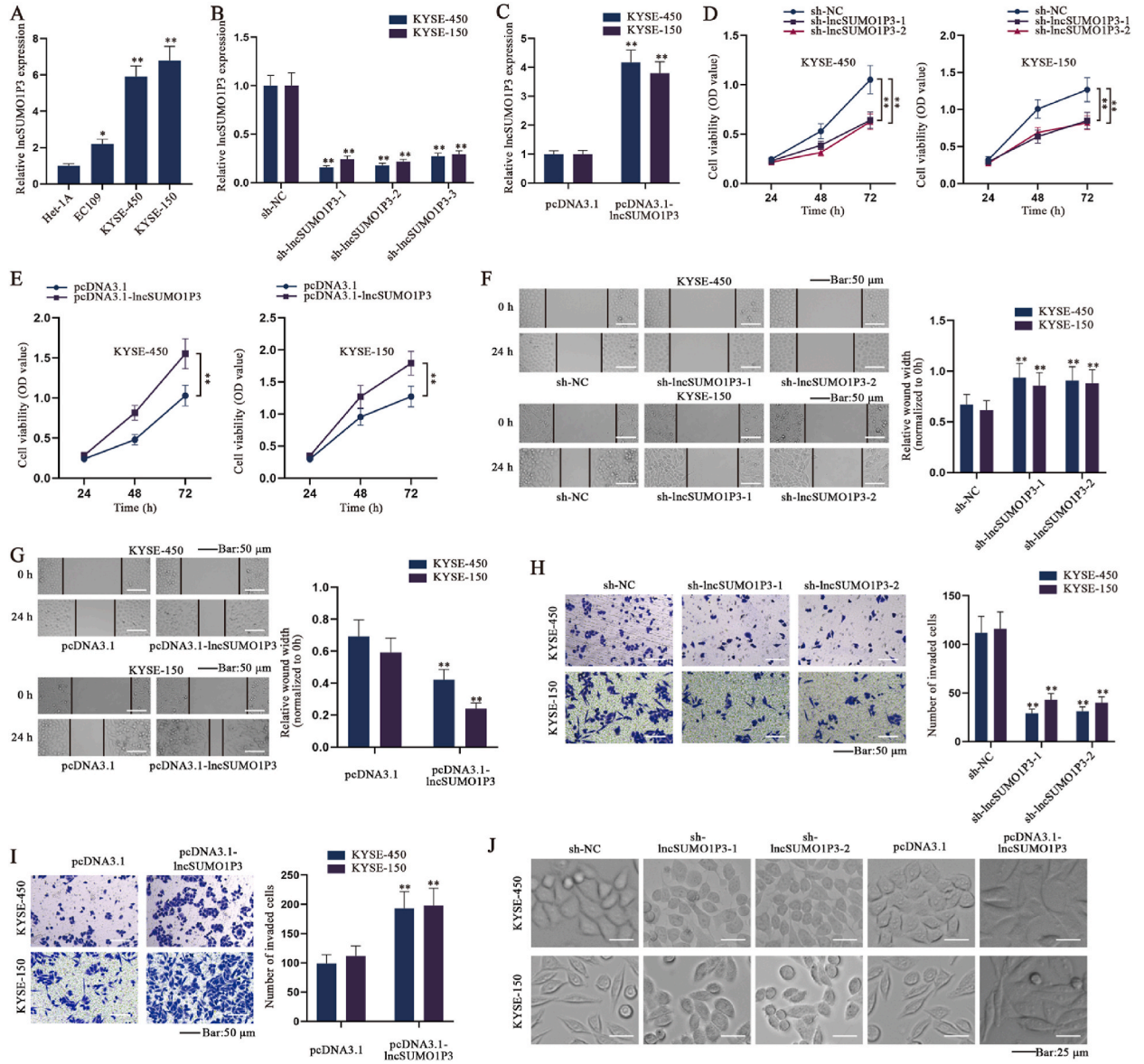

Fig. 1. LncSUMO1P3, overexpressed in ESCC cells, could aggravate the malignant behaviors of ESCC cells A. RT-qPCR examined lncSUMO1P3 expression in human ESCC cell lines and Het-1A cells. B–C. LncSUMO1P3 knockdown and overexpression efficiencies were tested in ESCC cell lines through RT-qPCR. D-E. CCK-8 assays were conducted after lncSUMO1P3 knockdown or overexpression to determine the influence of lncSUMO1P3 on ESCC cell proliferation. F-G. Wound healing assays were conducted after lncSUMO1P3 knockdown or overexpression to determine the influence of lncSUMO1P3 on ESCC cell migration. H–I. Transwell assays were conducted to determine the influence of lncSUMO1P3 silence or overexpression on ESCC cell invasion. J. Cell morphology was observed under optical microscope after lncSUMO1P3 knockdown or overexpression. Student's t-test was applied to analyze difference between two groups in Fig. 1C/1E/1G/1I while one-way ANOVA was applied for statistical analysis of multiple groups in Fig. 1A-B/1D/1F/1H. *P < 0.05, **P < 0.01.

detected to be abundant in Bio-miR-486-5p-WT, further confirming the binding correlation between lncSUMO1P3 and miR-486-5p (Fig. 2E). Moreover, it was manifested in RT-qPCR analysis that lncSUMO1P3 knockdown could hardly affect miR-486-5p expression, implying that lncSUMO1P3 could sponge miR-486-5p, rather than mediating miR-486-5p expression (Fig. 2F). Based on prediction of binding sequences between lncSUMO1P3 and miR-486-5p on DIANA, we constructed luciferase reporters and conducted dual luciferase reporter assay. It was observed that miR-486-5p mimics transfection could lessen the luciferase activity of pmirGLO-lncSUMO1P3-WT in ESCC cells while that of pmirGLO-lncSUMO1P3-Mut varied little (Fig. 2G). To conclude, lncSUMO1P3 could sponge miR-486-5p in ESCC cells.

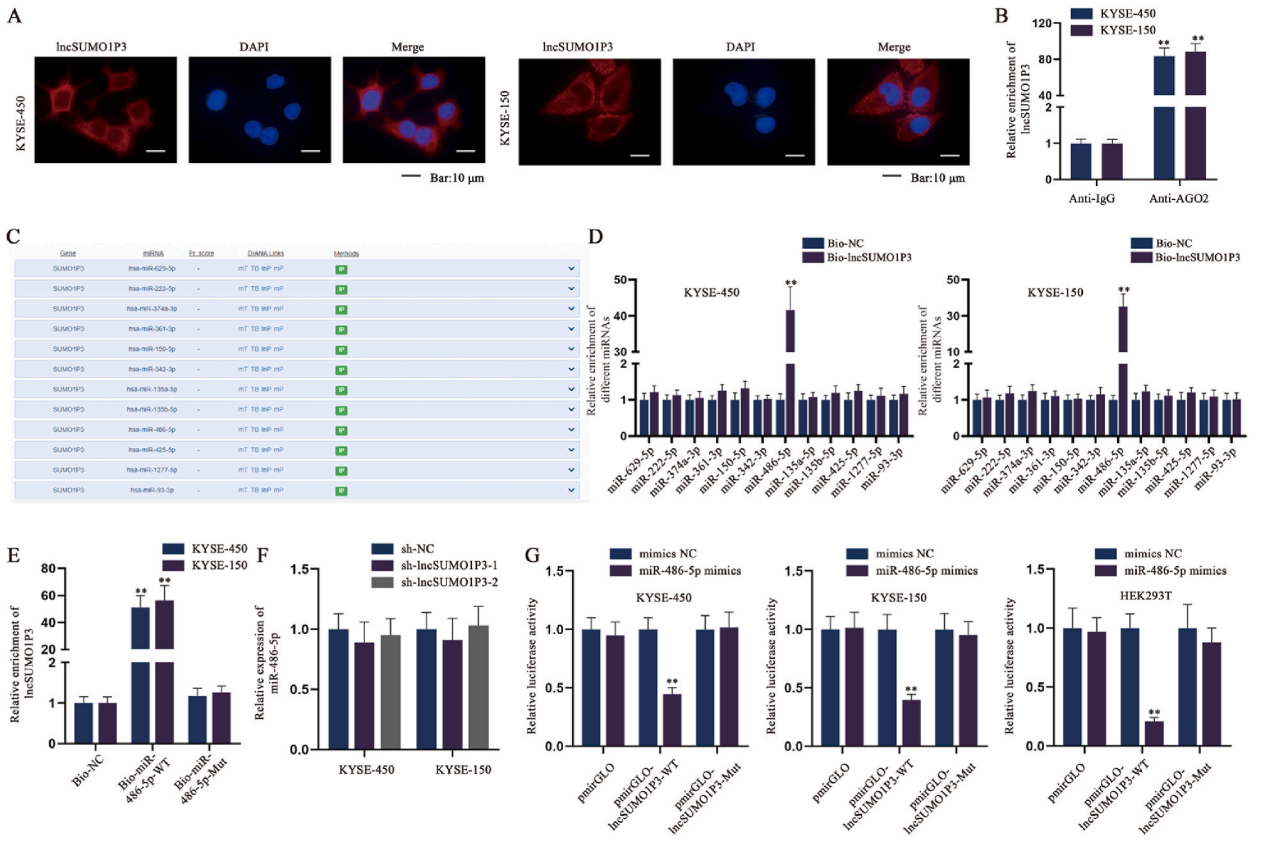

Fig. 2. LncSUMO1P3 could sponge miR-486-5p in ESCC cells A. FISH assay was carried out to determine the location of lncSUMO1P3 in KYSE-450 and KYSE-150 cells. B. Enrichment of lncSUMO1P3 in Anti-AGO2 precipitates was verified through RIP. C. Potential miRNAs that could bind with lncSUMO1P3 were predicted on LncBase Predicted v.2. D. RNA pulldown assay was conducted to determine the potential binding correlation between lncSUMO1P3 and predicted miRNAs. E. RNA pulldown was conducted with Bio-miR-486-5p-WT/Mut to verify the binding between lncSUMO1P3 and miR-486-5p. F. RT-qPCR analysis examined miR-486-5p expression in cells with or without lncSUMO1P3 depletion. G. Dual luciferase reporter assay was applied for determination of the binding between lncSUMO1P3 and miR-486-5p in KYSE-450, KYSE-150 and HEK293T cells. Student's t-test was applied to analyze difference between two groups in Fig. 2B. One-way ANOVA was applied for statistical analysis of multiple groups in Fig. 2E/2F while two-way ANOVA was applied in Fig. 2D/2G for statistical analysis. **P < 0.01.

3.3. LncSUMO1P3 upregulated CD151 by sponging miR-486-5p

To explore the integrated ceRNA network involving lncSUMO1P3 and miR-486-5p, candidate mRNAs of miR-486-5p were predicted on starBase (http://starbase.sysu.edu.cn/) under CLIP data strict stringency ≥5 and pan-cancer ≥11 (Fig. 3A). Referring to previous studies, CD151 has been proved to promote EMT in hepatocellular carcinoma [10] and ovarian cancer [11]. Also, CD151 has been verified to be closely associated with the metastasis and survival rate of ESCC patients [12]. Nevertheless, there is a lack of studies discussing the role of CD151 in ceRNA in ESCC. Therefore, CD151 was chosen as the target. As illustrated in RT-qPCR analysis, CD151 was notably downregulated after lncSUMO1P3 depletion (Fig. 3B–C). Moreover, it was manifested in RNA pulldown results that miR-486-5p could be substantially pulled down by Bio-CD151 3′UTR (Fig. 3D). In dual luciferase reporter assay, pmirGLO-CD151 3′UTR-WT and pmirGLO-CD151 3′UTR-Mut were constructed based on starBase prediction of the binding site between miR-486-5p and CD151, and were then respectively co-transfected with mimics NC or miR-486-5p mimics into ESCC cells and HEK293T cells. The luciferase activity of pmirGLO-CD151 3′UTR-WT was testified to be evidently weakened under miR-486-5p overexpression while that of pmirGLO-CD151 3′UTR-Mut hardly showed any difference under the same condition (Fig. 3E). Furthermore, KYSE-450 and KYSE-150 cells were transfected with different plasmids including sh-NC, sh-lncSUMO1P3-1, sh-lncSUMO1P3-1+inhibitor NC or sh-lncSUMO1P3-1+miR-486-5p inhibitor to confirm the impacts of lncSUMO1P3/miR-486-5p on CD151 expression. Interestingly, CD151 expression decreased with lncSUMO1P3 silence could only be partly reversed by miR-486-5p inhibitor co-transfection (Fig. 3F–G). In conclusion, lncSUMO1P3 could upregulate CD151 by sponging miR-486-5p in ESCC cells.

3.4. LncSUMO1P3 could regulate CD151 by recruiting PHF8

As we had discovered that miR-486-5p inhibitor could not completely reverse CD151 downregulation caused by lncSUMO1P3

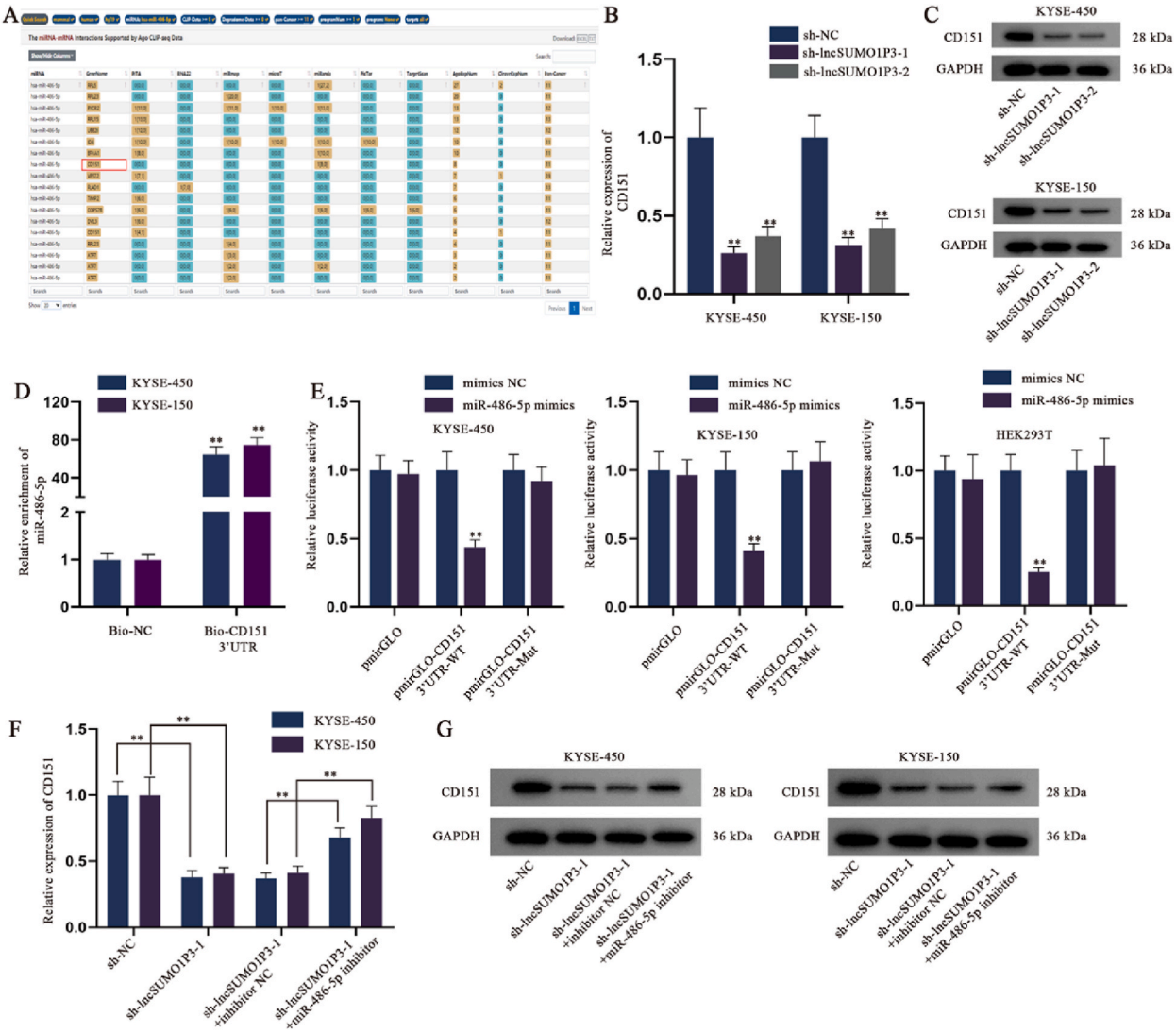

Fig. 3. LncSUMO1P3 upregulated CD151 by sponging miR-486-5p A. Potential downstream mRNAs of miR-486-5p were predicted on starBase (CLIP data strict stringency≥5 and pan-Cancer≥11). B. The expression of CD151 in KYSE-450 and KYSE-150 was tested by RT-qPCR after lncSUMO1P3 knockdown. C. Expression of CD151 was measured through WB after lncSUMO1P3 knockdown. D. MiR-486-5p pulled down by Bio-CD151 3′UTR in RNA pulldown assay was measured through RT-qPCR. E. Dual luciferase reporter assay verified the binding between CD151 3′UTR and miR-486-5p. F-G. The expression of CD151 in ESCC cells after transfecting sh-lncSUMO1P3-1, sh-lncSUMO1P3-1+inhibitor NC or sh-lncSUMO1P3-1+miR-486-5p inhibitor was tested by RT-qPCR and WB. Student's t-test was applied to analyze difference between two groups in Fig. 3D. One-way ANOVA was applied for statistical analysis of multiple groups in Fig. 3B/3F while two-way ANOVA was applied in Fig. 3E for statistical analysis. **P < 0.01.

silence, and based on the fact that lncSUMO1P3 also existed in nuclei of ESCC cells, it was assumed that lncSUMO1P3 might also modulate CD151 expression transcriptionally. By referring to previous studies, it was found that CD151 was once reported to be regulated by transcription factor SP1 in liver cancer [13]. However, it has not been elucidated whether CD151 could be affected by any other transcription factors in ESCC. Therefore, we searched on UCSC (http://genome.ucsc.edu/) for predicting potential transcription factors of CD151 (Fig. 4A). A series of experiments were also conducted to explore which transcription factor could bind with lncSUMO1P3 and regulate CD151 expression. The alterations in CD151 expression were detected through RT-qPCR after knockdown of potential transcription factors respectively. By analyzing RT-qPCR results, it could be confirmed that only PHF8 knockdown could dramatically reduce CD151 expression (Fig. 4B–C). PHF8 was thus selected as the research target. To further verify the influence of PHF8 on CD151 expression, we overexpressed PHF8 by transfecting pcDNA3.1-PHF8 into cells (Fig. 4D) and then examined the changes in CD151 mRNA through RT-qPCR, and the results indicated that CD151 mRNA level was augmented upon PHF8 overexpression (Fig. 4E). Afterwards, Western blot assay also showed that the protein levels of CD151 were also positively regulated by PHF8 (Fig. 4F). Mechanism assays were subsequently conducted for determination of the correlation between CD151 and PHF8.

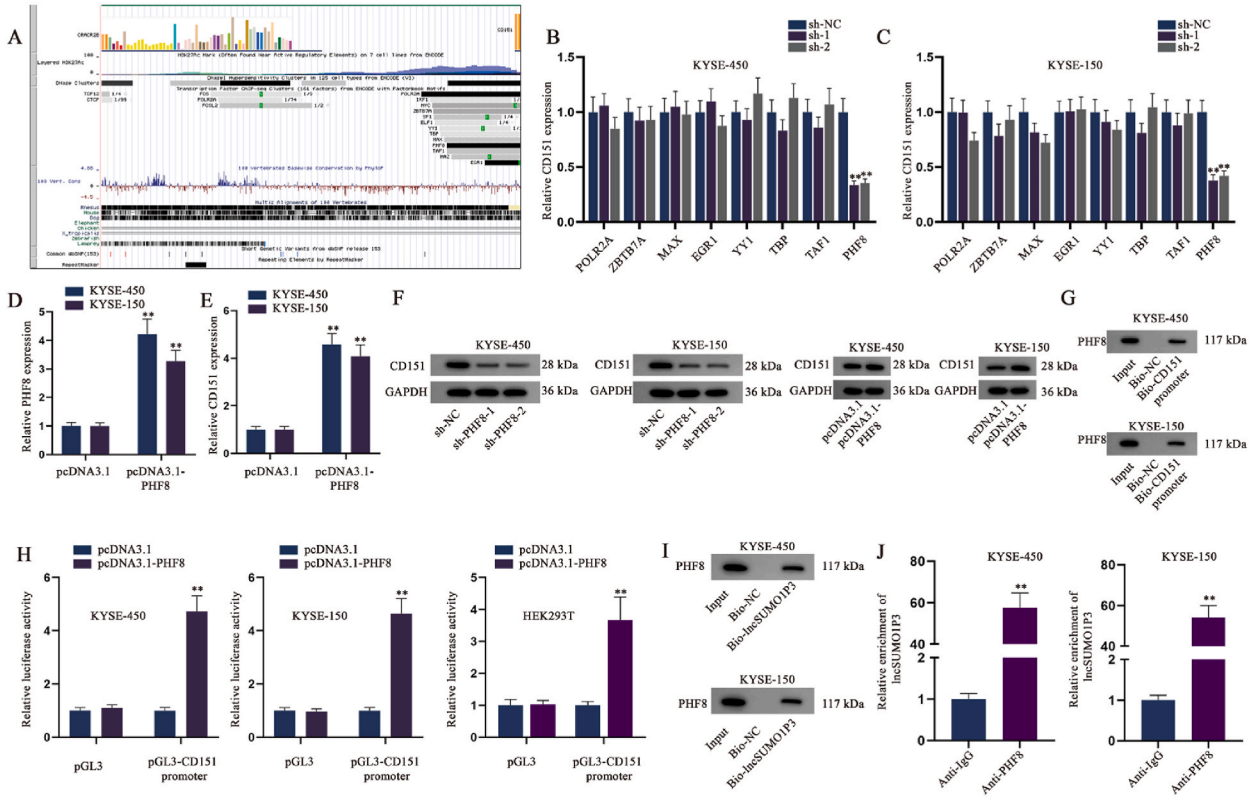

Fig. 4. LncSUMO1P3 could regulate CD151 by recruiting PHF8 A. Transcription factors of CD151 were predicted on UCSC. B–C. CD151 expression was evaluated by RT-qPCR after knockdown of predicted transcription factors. D. Overexpression efficiency of pcDNA3.1-PHF8 was detected through RT-qPCR. E. CD151 expression was examined through RT-qPCR after PHF8 overexpression. F. CD151 protein level was measured through WB in ESCC cells with PHF8 depletion or augment. G. DNA pulldown assay was done for evaluation of the binding between CD151 promoter and PHF8. H. Dual luciferase reporter assay was done for confirmation of the affinity between PHF8 and CD151 promoter. I. WB was conducted to measure PHF8 pulled down by Bio-lncSUMO1P3 in RNA pulldown assay. J. RIP assay verified the binding between PHF8 and lncSUMO1P3. Student's t-test was applied to analyze difference between two groups in Fig. 4D/4E/4J. One-way ANOVA was applied for statistical analysis of multiple groups in Fig. 4B while two-way ANOVA was applied in Fig. 4H for statistical analysis. $^{**}P < 0.01$.

According to DNA pulldown results, Bio-CD151 promoter largely pulled down PHF8, showing the combination between PHF8 and CD151 promoter (Fig. G). Subsequent dual luciferase reporter assay was carried out by transfecting pcDNA3.1 or pcDNA3.1-PHF8 and pGL3 or pGL3-CD151 promoter into ESCC cells and HEK293T cells. As a result, the luciferase activity of pGL3-CD151 promoter was much increased by pcDNA3.1-PHF8, elucidating that PHF8 could activate CD151 transcription via binding to CD151 promoter (Fig. 4H). Thereafter, to confirm whether lncSUMO1P3 was able to modulate CD151 via interacting with PHF8, we conducted RNA-Protein pulldown assay to detect the cohesion between lncSUMO1P3 and PHF8. We found that PHF8 was significantly pulled down by Bio-lncSUMO1P3 rather than Bio-NC, certifying that binding between lncSUMO1P3 and PHF8 (Fig. 4I), which was further confirmed by RIP results (Fig. 4J). Taken together, lncSUMO1P3 could modulate CD151 via interaction with PHF8.

3.5. CD151 could promote EMT of ESCC cells

Next, we intended to ravel out whether CD151 could affect EMT of ESCC cells. First, RT-qPCR was applied to test the efficiency of CD151 knockdown or overexpression in KYSE-450 and KYSE-150 cells (Fig. 5A–B). Subsequently, transfected cells were subjected to observation under optical microscope. The results showed that CD151 silencing significantly inhibited the EMT of ESCC cells, and the cell morphology changed from shuttle to round, while CD151 overexpression significantly promoted the EMT of ESCC cells, and the cell morphology changed from round to shuttle with loss of cell polarity (Fig. 5C). To further validate the influence of CD151 on EMT of ESCC cells, expression of EMT related genes namely, E-cadherin, N-cadherin, Claudin-1, Vimentin and Snail were measured via RT-qPCR and WB. It was found that the expression of E-cadherin and Claudin-1 was substantially enhanced while that of N-cadherin, Vimentin and Snail was dramatically decreased under the influence of CD151 silence (Fig. 5D–E). In addition, we overexpressed CD151 in cells and discovered that the expression of E-cadherin and Claudin-1 reduced while that of N-cadherin, Vimentin and Snail increased (Fig. 5F–G). To sum up, it was examined that CD151 could induce EMT of ESCC cells.

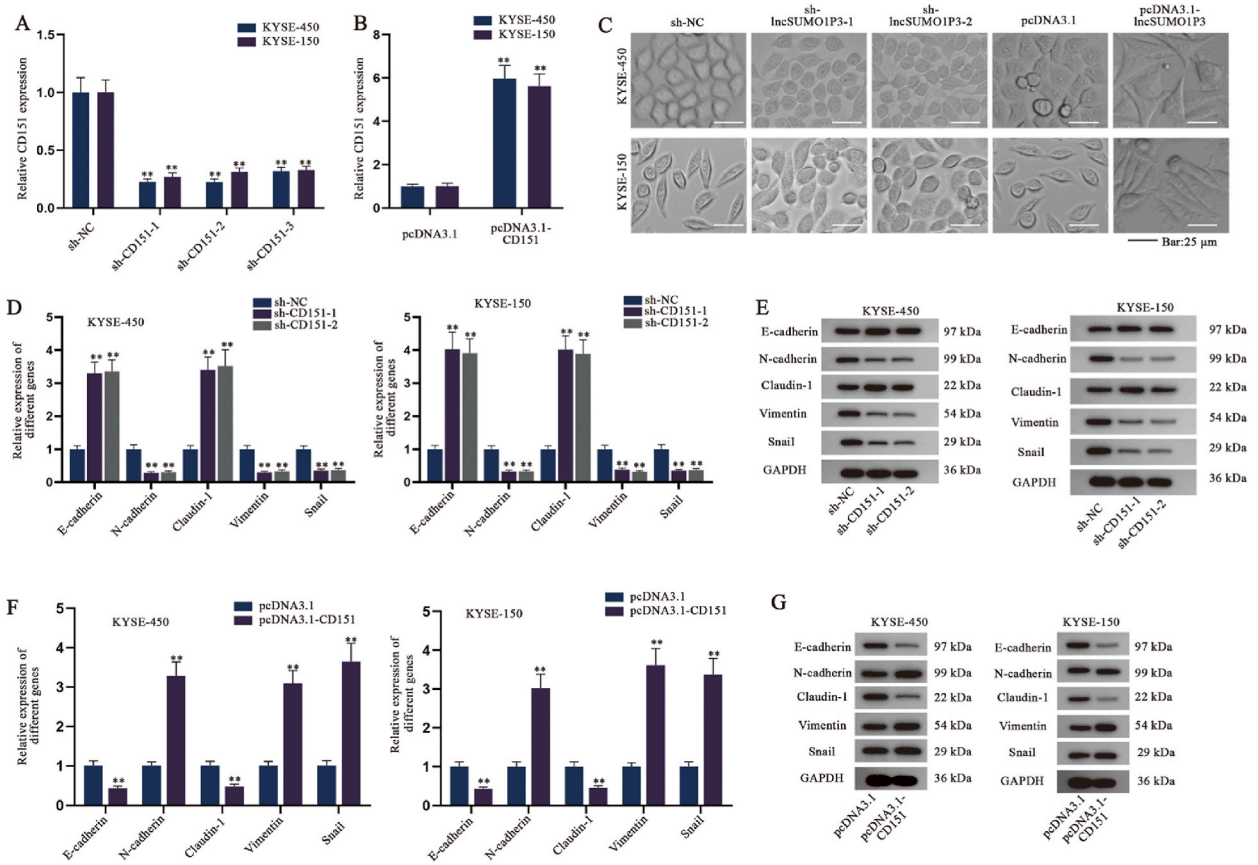

Fig. 5. CD151 could promote EMT of ESCC cells

A-B. RT-qPCR was implemented to test CD151 expression in ESCC cells with transfection of sh-CD151-1/2/3 or pcDNA3.1-CD151. C. Cell morphology was observed after CD151 knockdown or overexpression. D-E. Expression of EMT-related factors in ESCC cells with CD151 knockdown was evaluated through RT-qPCR and WB. F-G. Expression of EMT-related factors in ESCC cells with CD151 overexpression was examined through RT-qPCR and WB. Student's t-test was applied to analyze difference between two groups in Fig. 5B/5F while one-way ANOVA was applied for statistical analysis of multiple groups in Fig. 5A/5D. **P < 0.01.

4. Discussion

LncRNAs were RNA molecules longer than 200 nucleotides that were once regarded as "dark matter", which do not encode information about proteins [14]. However, lncRNAs were later identified with a myriad of molecular functions and associated with human diseases [15]. Previous studies have also verified that certain lncRNAs could affect the malignant behaviors of ESCC cells by acting as ceRNAs [3,6].

In this study, we discovered lncSUMO1P3 was abnormally overexpressed in ESCC cells, and inhibition of lncSUMO1P3 could obstruct EMT process while overexpressing of lncSUMO1P3 caused opposite effect, suggesting lncSUMO1P3 could aggravate cell malignant behaviors in ESCC. To further determine the relevant regulatory mechanism, we conducted bioinformatics analysis, FISH, RIP, pulldown and dual luciferase reporter assays, and discovered lncSUMO1P3 could competitively bind with miR-486-5p to release CD151 held up by miR-486-5p. However, we found that miR-486-5p inhibitor could only partially countervail the inhibitory effect of lncSUMO1P3 deficiency on CD151 expression, thus we postulated lncSUMO1P3 could regulate CD151 expression via other axis.

Referring to existing studies, lncRNA/Transcription Factor (TF)-mediated ceRNA network has been identified to be associated with gliobalastoma prognosis [16]. Herein, we assumed that lncSUMO1P3 could regulate CD151 through lncRNA/TF/gene triplets. To verify our assumption, we knocked down the expression of a number of transcription factors respectively in ESCC cells and found that only the knockdown of PHF8 could lead to obvious down-regulation of CD151. PHF8 is a histone demethylase that has been implicated in multiple pathological processes [17]. To further validate the influence of PHF8 on CD151 expression, we overexpressed PHF8 and found that CD151 was upregulated through RT-qPCR and WB. Meanwhile, we performed pulldown assay and dual luciferase reporter assay. The results testified PHF8 could interact with the promoter of CD151, thus regulating the translation of CD151. In addition, pulldown assay and RIP assay also proved that lncSUMO1P3 could bind with PHF8. Hence, we certified the regulatory axis of lncSUMO1P3/PHF8/CD151.

EMT refers to the transdifferentiation of epithelial cells into motile mesenchymal cells, which contributes to fibrosis and cancer

progression [18]. CD151 has been reported to affect EMT in HCC [10], in breast cancer [19], and in ovarian tumor [11], down-regulation of CD151 in colon cancer cells inhibits cell viability, migration and invasiveness【33767593】, but little evidence could prove CD151 affects EMT in ESCC. In this respect, we observed the ESCC cell morphology after CD151 depletion or augment. Additionally, we measured the expression of E-cadherin (cell adhesion molecules), Caludin-1 (epithelial cell marker protein) and EMT marker proteins (Vimentin, N-cadherin and Snail) by utilization of RT-qPCR and WB. The results fully explained that CD151 could induce EMT of ESCC cells.

To conclude, this study elucidated that lncSUMO1P3 could upregulate CD151 in ESCC cells and then induce EMT of ESCC cells by dual-axis of lncSUMO1P3/miR-486-5p/CD151 and lncSUMO1P3/PHF8/CD151. However, it still requires further study involving clinical samples and in-vivo experiments to better explore the therapeutic values.

Author contribution statement

Zhongwen Li: Conceived and designed the experiments; Wrote the paper.

Tingyou Zhang: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Guojun Yue: Performed the experiments.

Xin Tian: Analyzed and interpreted the data.

Ying Xu: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19110.

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