



A novel LINC00478 serves as a tumor suppressor in endometrial carcinoma progression

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Abstract

Background Long noncoding RNAs (lncRNAs) are involved in the pathogenesis and progression of various cancers, but their roles in endometrial cancer (EC) are largely unknown.

Methods The expressions of LINC00478 and PTBP1 in EC tissues were determined by RT-qPCR. Cell counting kit-8, flow cytometry and Transwell assays were executed for detecting the roles of LINC00478 in EC cells proliferation, migration and invasion. The mouse–xenograft models were established by subcutaneous injection in vivo. The interaction between LINC00478 and PTBP1 was confirmed by RNA pull-down assay and RNA-binding protein immunoprecipitation assay.

Results LINC00478 was significantly down-regulated in EC tissues while compared to that in their paracancerous samples, and a higher expression level of LINC00478 was negatively correlated with clinical progress of EC patients. Functional experiments in vivo and in vitro revealed that LINC00478 overexpression could dramatically retard the proliferation of EC cells, decrease the rate of colony formation, suppress the migration and invasion abilities of EC cells in vitro and inhibit tumor growth in vivo. Mechanistically, LINC00478 regulated the expression of PTBP1, a key factor in the Warburg effect, and affected the metabolic process of EC cells.

Conclusions LINC00478 acts as a tumor suppressor in EC by negatively controlling PTBP1 expression and influencing the Warburg effect, providing a potential biomarker and therapeutic target for patients with EC.

Keywords LINC00478 · Endometrial cancer · PTBP1 · Warburg effect

Introduction

Endometrial cancer (EC), one of the most common malignancies of the female reproductive system, is a global threat to women's health corresponding an increasing

morbidity year by year in the world (Sung et al. 2021; Chen et al. 2016). In recent decades, although accumulated evidence has demonstrated that continuous estrogen stimulation is a key factor for endometrial tumorigenesis (Liang and Shang 2013), there is still considerable part of EC patients whose tumor occurrence cannot be explained by abnormal levels of either estrogen or progesterone, indicating that the underlying mechanisms involved in

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EC progression are more complicated and confusing (Liu et al. 2018). At present, there is no ideal molecular marker for the diagnostic of EC, and additionally, many aspects of treatment remain mired in controversy. Therefore, it is of great practical significance to actively search for effective diagnostic markers and new therapeutic targets for patients with EC.

More than 90% of the nucleotides in human genome are transcribed into RNA, most of which are noncoding RNA (ncRNA) (Cech and Steitz 2014). Compared with the important role of coding RNA in the biological process, ncRNA was initially considered as “junk noise” (Slack and Chinnaiyan 2019). However, in recent years, a large number of studies have shown that ncRNA plays a critical regulatory role in physiological and pathological activities (Quinn and Chang 2016). Long noncoding RNAs (lncRNAs) are a subclass ncRNAs whose length are longer than 200 nt without coding potential (Batista and Chang 2013). In previous studies, lncRNA has been demonstrated to be systemic regulators in multiple biological processes, including epigenetic modification, gene transcription regulation and translation modification, and then affects cell cycle, cell differentiation, growth, aging and other biological behaviors (Yao et al. 2019; Engreitz et al. 2016). Their abnormal expression or functions are closely related to the occurrence and the development of a variety of complex diseases including malignant tumors (Lin et al. 2020; Yang et al. 2017). In addition, it has been reported that lncRNA has abnormal expression in different cancers and significant tissue specificity (Profiling Noncoding RNAs in Cancer 2016), which can affect the occurrence, infiltration, invasion and metastasis of cancer (Pichler et al. 2020; Olivero, et al. 2020). These characteristics make lncRNAs have great potential as markers of tumor diagnosis, progression and prognosis. Nevertheless, with the further research on lncRNAs, there are still numerous newly discovered lncRNAs in the role of tumor and the involved molecular mechanisms are not clear.

In the previous studies, we found a notably lower expression of LINC00478 in EC tissues through high-throughput RNA-sequencing data (Li et al. 2014). In the current study, we further enlarged clinical samples and validated that the LINC00478 expression in EC tissues was abnormally lower compared with the corresponding adjacent normal tissues. Higher expression of LINC00478 could significantly inhibit the clinical progress of EC patients. In addition, functional assays indicated that the overexpression of LINC00478 could significantly suppress the proliferation of EC cells, reduce the clone formation rate, and regulate the cell cycle, migration, invasion ability and tumorigenicity in athymic mice. Mechanistically, LINC00478 could directly bound to PTBP1, control the Warburg effect in EC cells and thus participate in the EC occurrence and development.

Materials and methods

Study subjects and cell culture

A total of 176 paired EC tissues and corresponding adjacent normal tissues were included in this study and described previously (Li et al. 2014). In short, 72 EC samples were collected from Southern Chinese patients at the affiliated hospitals of Guangzhou Medical University, and another 104 EC tissues were obtained from Eastern Chinese patients who underwent tylectomies at the affiliated hospitals of Soochow University. The clinical characteristics of the patients were recorded after receiving their written informed consent. The study was approved by the Institutional Review Board of Guangzhou Medical University. Human EC cell lines (HEC1A, HEC-1-B and Ishikawa) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and the human normal endometrial stromal cell line (hESC) was obtained from QunXian Biotechnology Co, Ltd (Guangzhou, China).

All cell lines were cultured in incubator (37 °C, 5% CO₂) and cultured in RPMI-1640 (Gibco, Shanghai, China) supplemented with 10% FBS (Gibco, Shanghai, China) and 1% penicillin–streptomycin (Invitrogen, CA, USA).

Lentivirus construction and cell transfection

The cDNA of LINC00478 was synthesized by iGene Biotechnology Co, Ltd (Guangzhou, China) and cloned into the lentivirus empty vector pEZ-Lv201. The lentiviral production and transduction were executed following previously published procedures (Lin et al. 2020). Briefly, the lentiviruses were generated in 293 T cells using the lentivirus packaging system according to the manufacturer's protocols. Two EC cell lines (HEC-1-B and Ishikawa) were transfected with control lentivirus and the overexpressed LINC00478 lentivirus, respectively. The cells were stably selected with puromycin for 2 weeks, and the drug-resistant cells were confirmed by qPCR assay and were used for further studies.

Cell proliferation assay

Cells infected with different lentivirus were seeded in 96-well plates (1000 cells/well). The cell proliferation was conducted by the cell counting Kit-8 (CCK-8, Engreen, Beijing, China) assay according to the manufacturer's instructions. The original medium was removed and replaced with culture medium containing 10% CCK-8 solution at 24, 48, 72, 96 h after cells were seeded. After 4-h incubation, the absorbance was measured at 450 nm using a microplate

reader (Bio-Rad, CA, USA). Each experiment was repeated in triplicate.

Colony formation assay

Cells with empty control or stably overexpressed LINC00478 were seeded into a 6-well plate (100 cell/well) and allowed to grow until visible colonies formed in complete growth media (about 2 weeks). The cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 30 min, washed, air-dried and photographed.

Cell cycle analysis

Flow cytometry was performed to analyze cell cycle progression as previously described (Qiu et al. 2016). Cells were plated at a concentration of 1×10^6 per 100 mm dish and permeabilized with 70% ethanol overnight at 4 °C. Subsequently, the cells were stained with 0.5 mL propidium iodide (PI) staining solution, and cellular DNA content was analyzed by FACSCanto™ Flow Cytometer (BD Biosciences, CA, USA).

Transwell assay

Cell migration and invasion were assessed by conducting the Transwell assay with Corning Transwell insert chambers (Corning) and BD BioCoat Matrigel Invasion Chamber (Becton Dickinson Biosciences), respectively. Approximately 1×10^4 (migration assay) or 1×10^5 (invasion assay) cells in 200 μ l of serum-free RPMI 1640 medium were seeded in the upper chamber, and 800 μ l medium with 10% FBS was filled in the lower compartment. After 24 h for migration assays or 48 h for invasion assays at 37 °C, cells on the underside of the membrane were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet (Invitrogen) in 20% ethanol. The number of cells were counted for nine randomly selected fields at $\times 200$ magnification using phase contrast microscopy (Olympus, Japan).

Quantitative real-time PCR

Total RNA was extracted from tissue samples or cultured cells using Biopure™ isolation reagent (Bio Scientific, Austin, TX, USA) followed by reverse transcription with the cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) on the ABI 7500 fast real-time PCR System (Applied Biosystems) to detect the expression level of a gene of interest. The relative expression of each gene normalized to β -actin was

calculated by the $2^{-\Delta\Delta C_t}$ method. All primers used in the experiments are shown in supplementary Table S1.

Western blot analysis

Cells were lysed in RIPA buffer, and the protein concentrations were normalized with a BCA assay kit (Thermo Fisher Scientific, MA, USA). Then, 20 μ g of protein was subjected to 10% SDS-PAGE gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes blocked with nonfat milk were incubated with primary antibodies (PTBP1: cat#ab133734; Abcam), HRP-conjugated secondary antibodies, and visualized using the ECL system (Thermo Fisher Scientific, Rochester, NY).

Subcellular localization of LINC00478

For subcellular fractionation experiment, nuclear and cytoplasmic RNAs from EC cells were extracted using a Nuclear/Cytosol Fractionation kit (Biovision) according to the manufacturer's instructions. RNAs were converted to cDNA and analyzed by qPCR assay, and β -actin was used for normalization. Also, fluorescence in situ hybridization (FISH) assay was further performed with a Ribo™ lncRNA FISH Kit (RiboBio, Guangzhou, China). FISH probes against LINC00478 were designed and synthesized by RiboBio (RiboBio, Guangzhou, China). Briefly, cells were fixed with 4% paraformaldehyde at RT for 15 min, permeabilized in 0.5% Triton X-100 for 10 min on ice. Hybridization was carried out overnight in a humidified chamber at 37 °C in the dark. Cellular nuclei were counterstained with DAPI. Immunofluorescence was observed by a confocal microscope (Nikon, Tokyo, Japan). U6 were used as the nuclear markers.

RNA pull-down assay

Briefly, the in vitro biotin labeled of LINC00478 biotin probe and control probe was transfected into EC cells. Then, cells were lysed and cultivated with probe-coated beads at 4 °C. The RNA–protein complexes were pulled down by Pierce™ Streptavidin Magnetic Beads (Thermo Scientific) for 15–30 min at room temperature with agitation, incubated with protein lysates and then mixed with the RNA–beads complex for 30–60 min at 4 °C with agitation or rotation. The pull-down complexes were then washed and boiled at 95–100 °C for 5–10 min, followed by western blot assay.

RNA immunoprecipitation (RIP)

The Magna RIP™ RNA-binding Protein Immunoprecipitation Kit (Millipore, Massachusetts, USA) was utilized to perform RIP assay following the manufacturer's protocols.

After washing logarithmic phase EC cells with precooling PBS, the cells were lysed and mixed with RIP buffer. The protein A / g magnetic beads were mixed with anti-PTBP1 antibody or mouse IgG at 37 °C. Then the lysate was mixed with globin complex were incubated overnight at 4 °C. After washing 3 times and incubating with proteinase K digestion buffer, the coprecipitated RNAs were purified, extracted and analyzed by RT-qPCR to assess the enrichment of PTBP1 to LINC00478.

Glucose absorption and lactic acid production assay

Cell glycolytic metabolism was analyzed using Glucose Uptake Colorimetric Assay kit (Biovision) and Lactate colorimetric assay kit (Biovision) according to the manufacturer's instructions. For analysis, the supernatant was collected after centrifuging. The absorbance was determined at a wavelength of 450 nm with a spectrometer, and all data were expressed as a percentage of control.

RNA-sequencing (RNA-seq) processing

The total RNAs were extracted from overexpressed LINC00478 and empty control Ishikawa cells with TRIzol reagent. A NanoDrop (NanoDrop; Thermo Fisher Scientific, Inc.) was used to measure the quantity and quality of RNA. The transcriptome sequencing was prepared using HISAT2 for Illumina® according to the manufacturer's protocols (Promegene Biotechnology Co., Ltd, Guangzhou). Raw reads were aligned to the human genome GRCh38 by Bowtie2, and the gene values were quantified using the expected number of Fragments Per Kilobase of transcript sequence per millions base pairs sequenced (FPKM). Differentially expressed genes were identified according to $\log_2(\text{FoldChange}) > 1$ and $P < 0.05$ by R package EdgeR (version 3.6.3).

Tumorigenicity assays in nude mice

4–6-week-old BALB/c female nude mice purchased from Nanjing Biomedical Research Institute of Nanjing University were used to conduct the subcutaneous model. EC cells transfected with empty control or LINC00478 overexpression (2×10^7 cells/ml) was subcutaneously injected into the neck of each mouse that was randomly divided into two groups (with 5 mice each group). The tumor diameters were measured every 3 days with calipers, and the tumor volumes were calculated according to the following formula: $[(\text{width})^2 \times \text{length}] / 2$. After injection of 4 weeks, the mice were euthanized via dislocation of cervical vertebra method and the tumors were dissected and weighed. All procedures were implemented with the approval of the Animal Care and Use Committee of Guangzhou Medical University.

Bioinformatics analysis of LINC00478

Differences in gene expression of LINC00478 in pan-cancer and normal tissues were analyzed from the TIMER database (<http://timer.cistrome.org/>). To investigate the association of LINC00478 with immune infiltration in EC, we downloaded the gene expression profiles of EC from the TCGA data portal (<https://portal.gdc.cancer.gov/>). We applied the single-sample gene set enrichment analysis (ssGSEA) and CIBERSORT methods to estimate the infiltration levels of various immune cells in the TCGA-UCEC samples. The immune score, tumor purity, estimate score and stromal score of each cancer sample were calculated with the ESTIMATE algorithm (Yoshihara et al. 2013). Pearson correlation analysis was used to examine the correlation between LINC00478 and immune infiltration level.

Statistical analysis

The Chi-square test was used to assess the differences of LINC00478 expression distribution in demographical and clinical characteristics. The differences in gene expression, colonies number levels and cells' ability to invade and migrate were analyzed using the student's *t*-test. The correlation between LINC00478 expression and HHIP or clinical features was tested by spearman rank correlation. Repeated measure ANOVA test was performed to analyze the mean tumor volume of different group, as well as the different transfectants proliferation. All experiments in vitro were performed with independent triplicates. All tests were two-sided by using the SAS software (version 9. 3; SAS Institute), and the differences were considered statistically significant with $P < 0.05$.

Results

LINC00478 is lowly expressed in EC tissues and EC cell lines

The baseline information of the studied patients had been described previously (Li et al. 2014) and is presented in Table S2. As shown in Fig. 1, LINC00478 expressions were significantly decreased in EC tissues compared with that in adjacent normal tissues in Guangzhou set (Fig. 1A, $P < 0.01$), and this meaningful result was further validated in Suzhou set (Fig. 1B, $P < 0.01$). In addition, by using the TCGA-UCEC, GSE106191 and GSE17025 datasets to enlarge the sample size, we also confirmed that the expression of LINC00478 had significant reduced in EC tissues

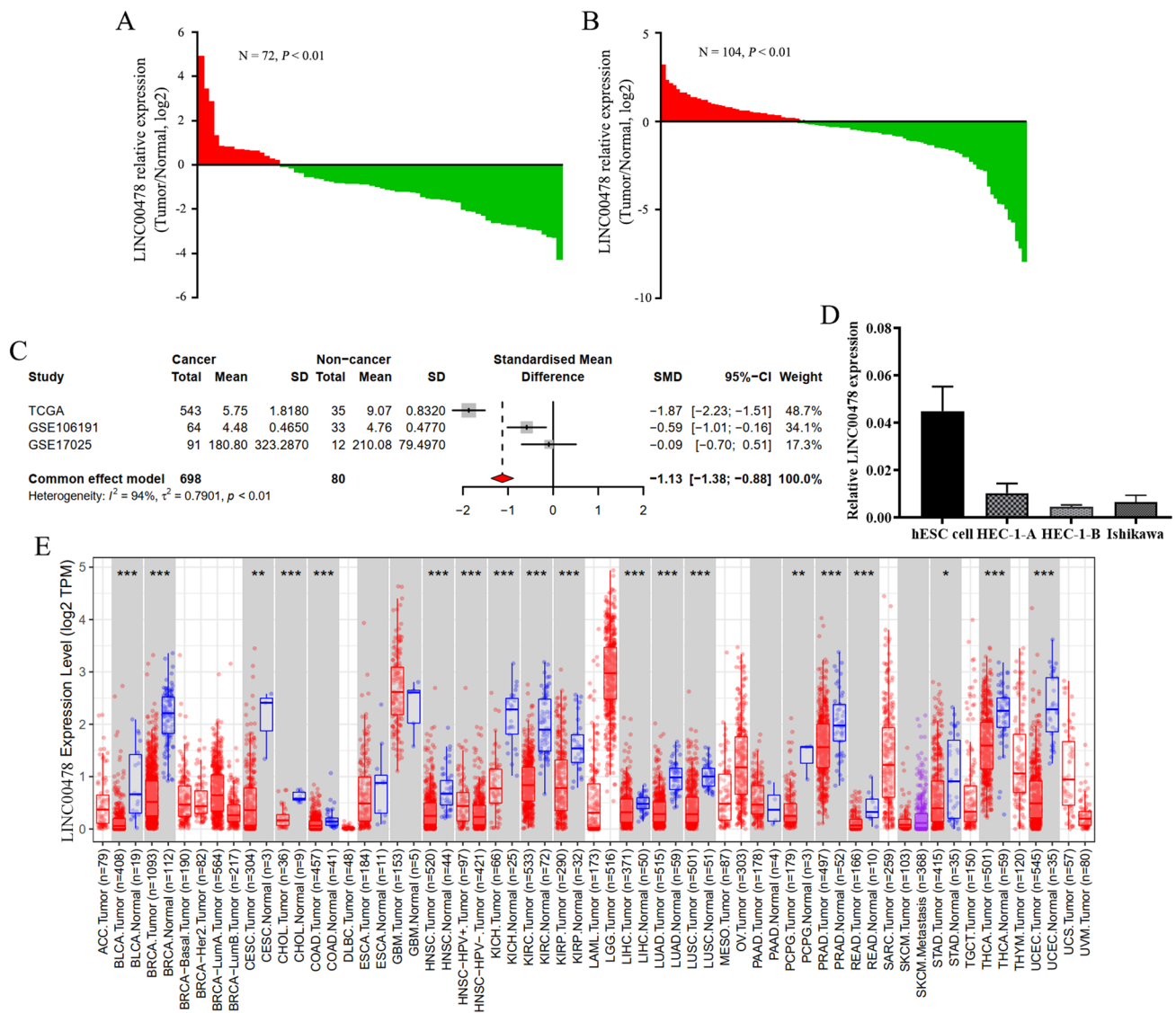


Fig. 1 LINC00478 is lowly expressed in EC tissues. **(A)** Quantification of LINC00478 expression in 72 paired EC and normal tissues in Guangzhou set. **(B)** Quantification of LINC00478 expression in 104 paired EC and normal tissues in Suzhou set. **(C)** Meta-analysis of

LINC00478 by using TCGA and GEO datasets. **(D)** Relative expression levels of LINC00478 in hESC cells and EC cell lines. **(E)** Differential analysis of LINC00478 expression in TCGA pan-cancer

than in paired adjacent normal tissues with systematic analysis (Fig. 1C, $P < 0.01$).

The expression levels of *LINC00478* in the hESC normal endometrial stromal cell line and EC cell lines including HEC1A, HEC-1-B and Ishikawa were further analyzed. Consistent with the previous results, LINC00478 expression levels were lower in EC cell lines than in hESC cells (Fig. 1D). Since HEC-1-B and Ishikawa showed the lowest levels of LINC00478, we chose these two cell lines for subsequent experiments. Moreover, the TIMER2.0 database (<http://timer.cistrome.org/>) was used to examine the LINC00478 expression levels in pan-cancers. The expression level of LINC00478 was significantly lower in almost

all tumor types in TCGA compared to normal samples (Fig. 1E).

LINC00478 is significantly associated with tumor stage

In order to analyze the association between LINC00478 expression and clinicopathological features, patients were divided into high-expression group and low-expression group, according to the median value of *LINC00478* level. As shown in Table 1, of note, the proportion of *LINC00478* high expression in FIGO advanced stage was significantly lower than that in FIGO early stage ($P = 0.033$), and the fact

Table 1 Correlation between *LINC00478* expression and clinicopathological characteristics in patients with endometrial cancer

Characteristics	<i>LINC00478</i>		P-value ^c	P-value ^d
	Lower-expression <i>LINC00478</i> group, N ^a (%)	High-expression <i>LINC00478</i> group, N ^a (%)		
Age (years)				
≤ 65	47(53.4)	39(44.3)	0.228	0.452
> 65	41(46.6)	49(55.7)		
Age at menarche (years)				
≤ 11	5(5.7)	4(4.6)	0.874	0.669
11–16	58(65.9)	61(69.3)		
≥ 16	25(28.4)	23(26.1)		
Menopausal status				
Premenopausal	25(28.4)	23(26.1)	0.735	0.817
Postmenopausal	63(71.6)	65(73.9)		
Family history of cancer				
Positive	5(5.7)	8(9.1)	0.387	0.957
Negative	83(94.3)	80(90.9)		
FIGO ^b stage				
I	62(70.5)	75(85.2)	0.033	0.005
II	7(7.9)	6(6.8)		
III	19(21.6)	7(8.0)		
Histologic type				
Endometrioid	79(89.8)	78(88.6)	0.808	0.825
Non-endometrioid	9(10.2)	10(11.4)		
Grade				
G1	31(35.2)	40(45.5)	0.310	0.296
G2	50(56.8)	44(50.0)		
G3	7(8.0)	4(4.5)		

^aN Number of patients^bFIGO The International Federation of Gynecology and Obstetrics^cChi-squared test P value^dSpearman correlation analysis P value

implied that increased *LINC00478* expression was negatively associated with EC stage ($r = -0.211$, $P = 0.005$). These data indicated that *LINC00478* functions as a tumor suppressor and correlates with the progression of EC.

LINC00478 inhibits EC cell growth and metastasis in vitro and in vivo

To ascertain that whether *LINC00478* functions as a tumor suppressor lncRNA, we constructed two *LINC00478*-overexpression EC cell lines, HEC-1-B and Ishikawa cells, and analyzed the roles of *LINC00478* in regulating the cell proliferation, colony formation ability, migration,

invasion and cell cycle progression. As shown in Fig. 2, the CCK-8 assay shown that overexpression of *LINC00478* significantly inhibited the EC cells proliferation (Fig. 2A). Colony formation assay in plates also confirmed that ectopic expression of *LINC00478* notably reduced the number of colonies, suggesting that up-expressed *LINC00478* could suppress the colony formation ability of EC cells (Fig. 2B). In addition, flow cytometry was applied to analyze differences in cell cycle distributions following *LINC00478* alteration. The results showed that overexpressed *LINC00478* increased the percentage of HEC-1-B cells in S phase and reduced the percentage in G0/G1 phase (Fig. 2C), demonstrating that *LINC00478* overexpression suppressed cell cycle progression in G0/1 transition. These findings were not observably existed in Ishikawa cells. Furthermore, transwell assays demonstrated that the cell ability to migrate or invade was prominently suppressed in cells with overexpressed *LINC00478* than that in empty control (all $P < 0.05$, Fig. 2D, E). To validate the anti-tumor effect of *LINC00478* in vivo, we then established subcutaneous xenograft EC mouse models and found that enforced expression of *LINC00478* drastically reduced the volumes and weights of xenograft tumors (all $P < 0.05$, Fig. 2F, G). To sum up, these data indicated that *LINC00478* overexpression restrains the cell proliferation and metastasis of EC.

LINC00478 interacted with PTBP1 and was negatively associated with PTBP1 expression

The subcellular localization of lncRNAs is closely associated with their biological functions. We observed that *LINC00478* was mainly expressed in the cytoplasm, and partially in the nucleus, of HEC-1-B and Ishikawa cells (Fig. 3A, B). This indicated that *LINC00478* might function at both transcriptional and posttranscriptional levels.

Previous studies have identified that lncRNAs can play a functional role in disease by interacting with coding genes and regulating their target genes expression (McIntyre et al. 2022). Therefore, we utilized the bioinformatic tools including ENCORI, The Encyclopedia of RNA Interactomes platform (Li, et al. 2014) and AnnoLnc, a web server for systematically annotating novel human lncRNAs (Hou et al. 2016) to predict potential molecular targets associated with *LINC00478*. The results revealed 68 potential target molecules of *LINC00478* using ENCORI database (Supplementary Table S3) and 7 potential target molecules of *LINC00478* from the AnnoLnc database (Supplementary Table S4). Based on the overlapping results, we identified 4 common potential target molecules of *LINC00478*, which were FMR1, LIN28B, CSTF2T and PTBP1 genes (Fig. 3C). We then analyzed the associations of *LINC00478* expression with these 4 candidate target genes and found

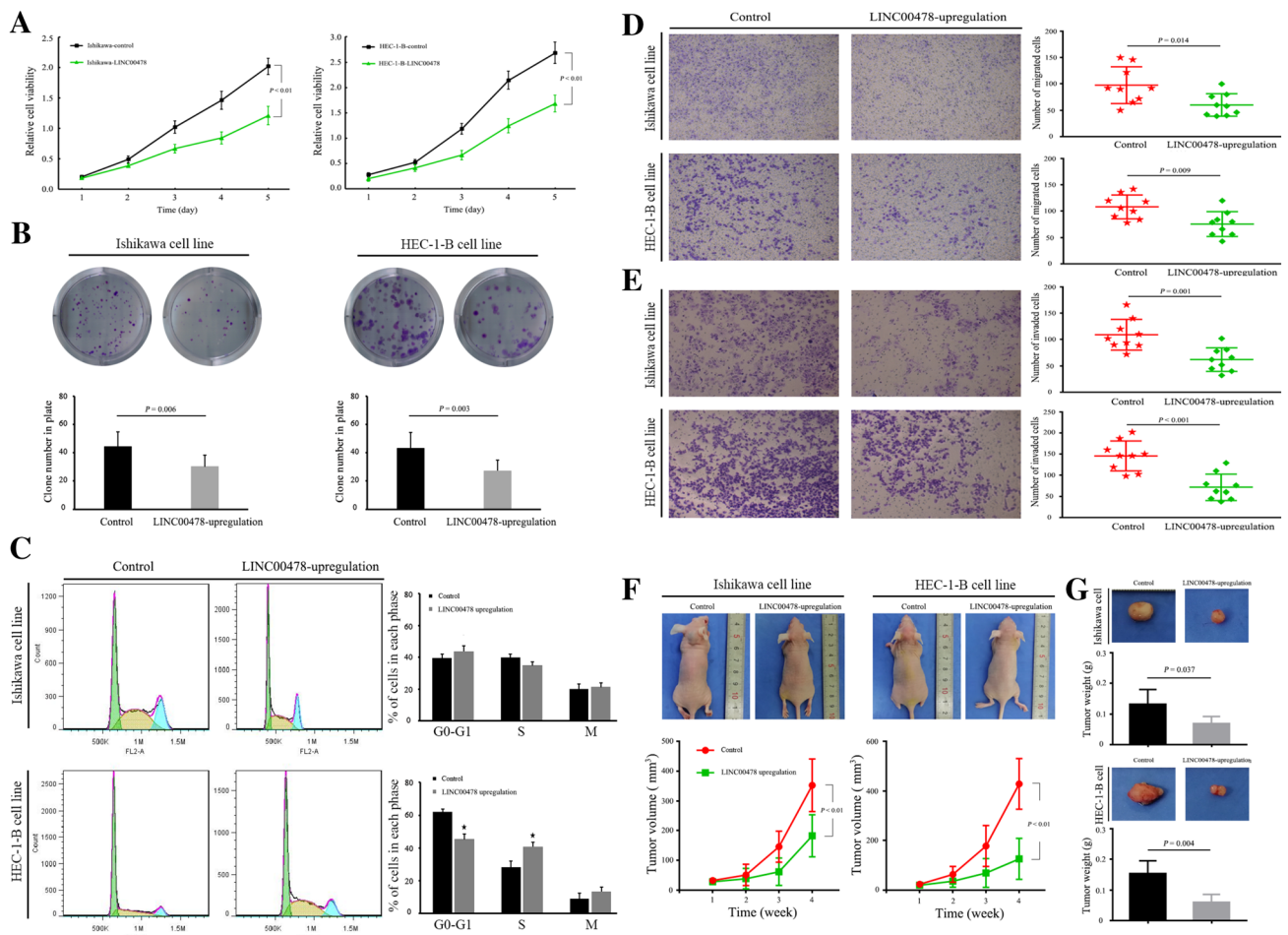


Fig. 2 LINC00478 inhibits EC cell growth and metastasis in vitro and in vivo. **(A)** Cell viability of indicated Ishikawa and HEC-1-B cells was measured by CCK-8 assay. **(B)** Plate clone formation assay. **(C)** The cell cycle was evaluated by flow cytometry. Left panel: X-axis: cell number; Y-axis: DNA content. **(D, E)** Cell migration and invasion of indicated Ishikawa and HEC-1-B cells were measured by

transwell migration and Matrigel invasion assays, respectively. **(F, G)** Representative images of tumors and tumor weight in nude mice; Growth curve of transplanted tumor in mice of each group ($n=5$) and mass of mice subcutaneous xenograft after ectopic expression of LINC00478 at day 28

that overexpression of LINC00478 significantly reduced the expression levels of PTBP1 at both mRNA and protein levels (Fig. 3D, E), suggesting that LINC00478 might interact with PTBP1 and modulate its expression. RNA pull-down assay confirmed that LINC00478 was specifically bound to PTBP1 protein (Fig. 3F), and the interaction between PTBP1 and LINC00478 was further assessed by RIP. RIP assay showed that LINC00478 could directly bind PTBP1 (Fig. 3G).

Moreover, the expression levels of PTBP1 gene were significantly up-regulated in EC tissues compared to adjacent non-cancerous tissues ($P < 0.01$, Fig. 3H). Interestingly, the expression of LINC00478 was negatively correlated with PTBP1 expression in EC tissues ($r = -0.711$, $P < 0.01$, Fig. 3I). Based on these results, we speculated that LINC00478

might regulate EC progression by negatively targeting the PTBP1 protein.

Up-expression of LINC00478 improving Warburg effect in EC cells

In contrast to normal cells metabolism, cancer cells produce more lactic acid and less adenosine triphosphate under aerobic conditions. This process is called aerobic glycolysis or “Warburg effect.” Recently, studies have reported that by reducing PTBP1 expression can be improving the Warburg effect of tumor cells (Takai et al. 2017). Given the characteristics of Warburg effect in tumor cells and aforementioned findings, we hypothesized

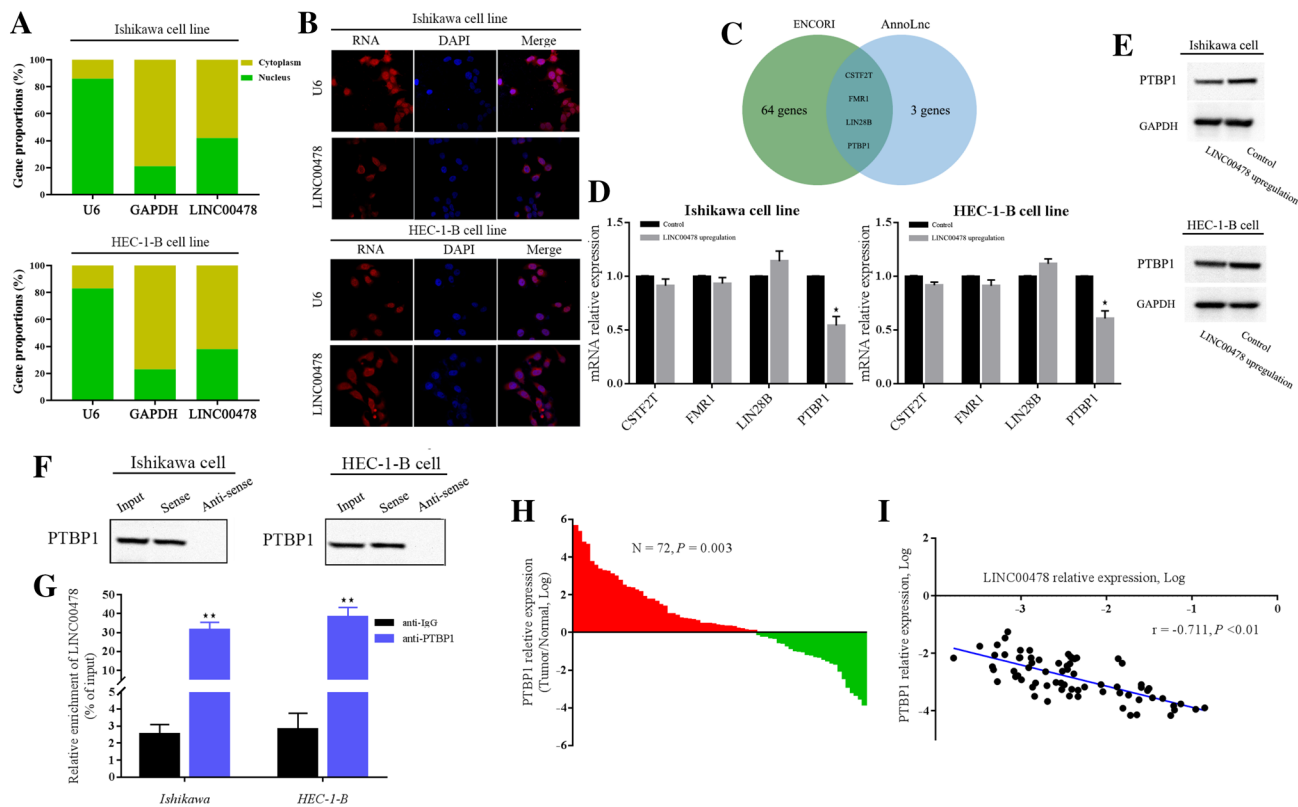


Fig. 3 LINC00478 interacted with PTBP1 and was negatively associated with PTBP1 expression. (A) LINC00478 subcellular localization detected by nuclear/cytoplasmic fractionation assay. (B) LINC00478 subcellular localization detected by FISH assay. (C) Venn diagram showing the predicted target of LINC00478 identified using ENCORI and AnnoLnc database. (D) qRT-PCR results confirmed the differ-

entially expressed genes induced by LINC00478. (E) Western blot assay for comparisons of PTBP1 protein levels between up-expressed LINC00478 and empty control EC cells. (F, G) LINC00478 interacted with PTBP1 in EC cells, as revealed by RNA pull-down and RIP assay, respectively. (H) Expression of PTBP1 in EC tissues. (I) Correlation analysis of LINC00478 with PTBP1 in EC tissues

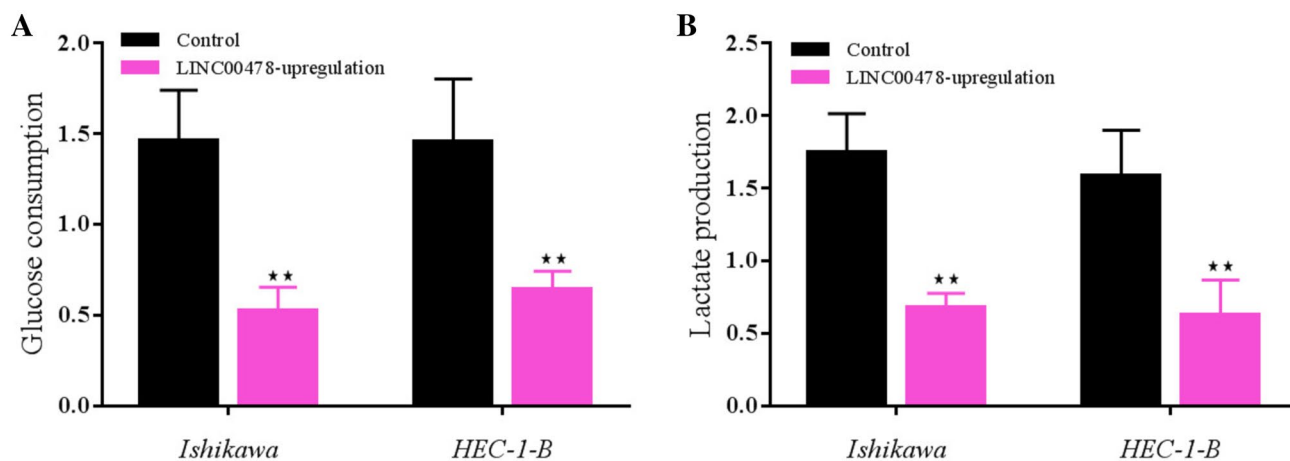


Fig. 4 Up-expression of LINC00478 improving Warburg effect in EC cells. (A) glycoese consumption assay. (B) lactate production assay. Measurement data were described as mean ± standard deviation. The

independence sample *t* test was used for comparison between the two groups. The cell experiment was repeated three times independently

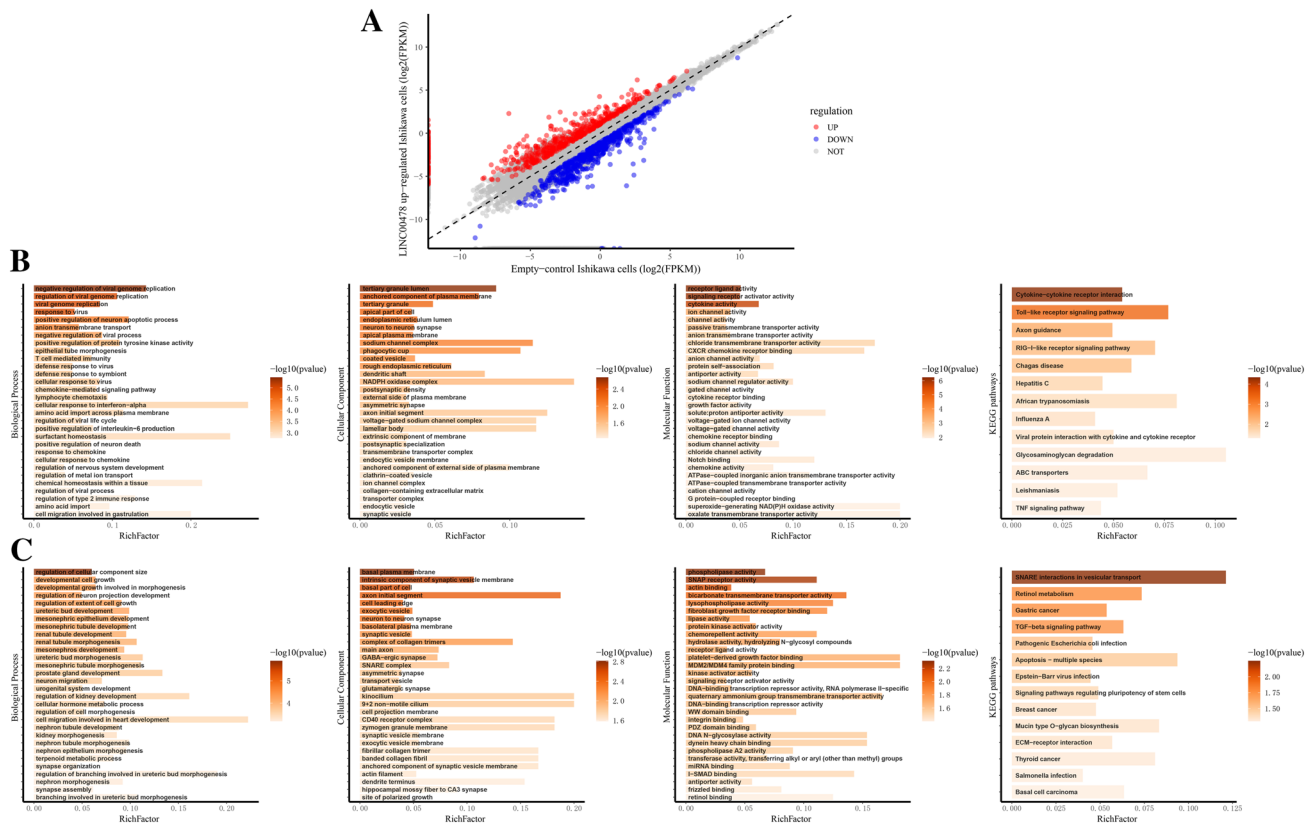


Fig. 5 RNA-Seq results induced by LINC00478 and their functional enrichment analysis. **(A)** A scatter plot of differentially expressed genes between Ishikawa cells with up-expressed LINC00478 and empty control. **(B)** GO and KEGG enrichment analysis with up-

expressed genes. **(C)** GO and KEGG enrichment analysis with down-expressed genes

whether LINC00478 could affect the aerobic glycolysis in EC cells. As shown in Fig. 4, compared to the empty control cells, the cells with highly expressed LINC00478 had lower glucose consumptions and less lactate productions.

The above results suggested that LINC00478 could change the glycolysis of EC cells through modulating PTBP1 and downstream pathway, and played a crucial role in metabolic reprogramming.

LINC00478 regulates multiple signaling pathways associated with cell growth and metastasis

To further understand the detailed mechanism of LINC00478-regulated tumor growth and metastasis, we performed RNA-seq to filtrate the genes regulated by ectopic expression of LINC00478 and found that there were 567 up-regulated and 634 down-regulated genes in LINC00478 up-regulated Ishikawa cells compared with controls (Fig. 5A). As presented in Fig. 5B, C, Gene Ontology (GO) analysis indicated that LINC00478 could regulate a series of

biological processes that are associated with tumor progression, such as developmental cell growth, cellular hormone metabolic process, cell growth and cellular hormone metabolic process. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis also presented that there were several signaling pathways closely associated with carcinogenesis and tumor progression enriched, including TGF-beta signaling pathway, glycosaminoglycan degradation and Toll-like receptor signaling pathway.

Correlation between LINC00478 and the infiltration of immune cell subtypes

Immune destruction has been recognized as an emerging hallmark of cancer. Previous studies have shown that abundant inflammatory cells infiltrate the EC tissues, not only around the tumor but also in the tumor matrix, and that the complex tumor immune microenvironment may influence the treatment efficacy and clinical outcome (Lopez-Janeiro et al. 2022). Based on the

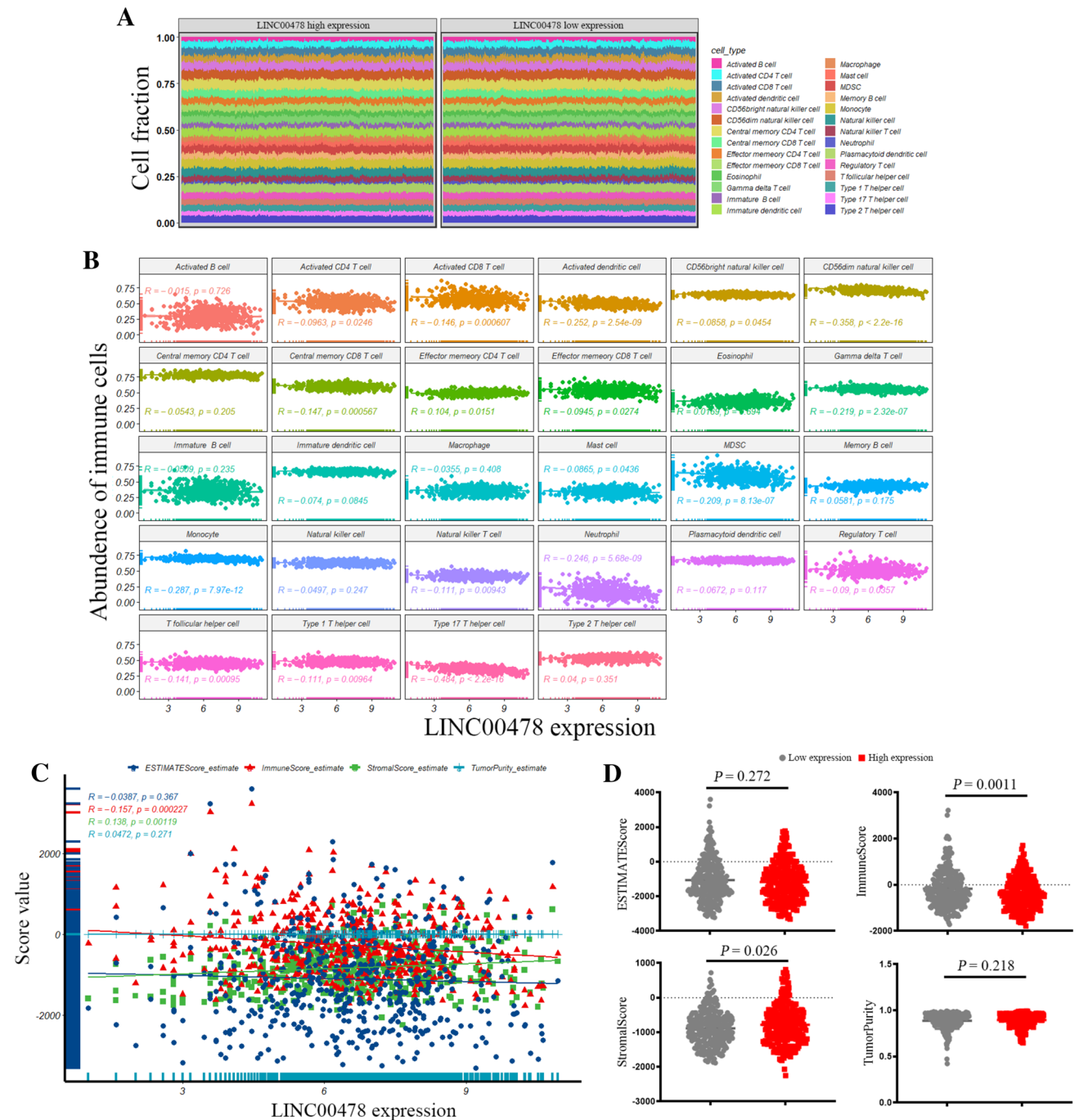


Fig. 6 Correlation between LINC00478 and tumor immune cell infiltration. **(A)** Difference in the proportion of each immune cell type between the low-expression and high-expression groups of LINC00478, calculated using the ssGSEA method. **(B)** Relationship between the LINC00478 expression and immune cell infiltration. **(C)** Using ESTIMATE's algorithm, the estimate score, the immune score,

stromal score and tumor purity of each sample gene were quantified, and their correlations with LINC00478 expression were analyzed. **(D)** The comparisons of the immune score, stromal score and tumor purity between the low-expression and high-expression groups of LINC00478

functional enrichment analysis results of LINC00478, we hypothesized that this lncRNA might be involved in tumor immunity. To test this hypothesis, we examined the correlation between LINC00478 expression and the

infiltration of various immune cell subtypes in EC using the TCGA data. As shown in Fig. 6A, there was a significant difference in the infiltration of immune cells between the LINC00478 high-expression and low-expression

groups. Notably, LINC00478 expression was negatively correlated with activated CD4 T cell, activated CD8 T cell, activated dendritic cell, CD56bright natural killer cell, CD56dim natural killer cell, central memory CD8 T cell, effector memory CD8 T cell, gamma delta T cell, mast cell, MDSC, monocyte, natural killer T cell, neutrophil, regulatory T cell, T follicular helper cell, type 1 T helper cell and type 17 T helper cell, respectively. Conversely, a positive correlation was observed between LINC00478 expression and effector memory CD4 T cell (Fig. 6B). Furthermore, after applying the ESTIMATE algorithm, we found that LINC00478 expression was negatively associated with immune score and positively associated with stromal score (Fig. 6C). Compared with the low-expression group, the high-expression group of LINC00478 had lower immune score but higher stromal score (Fig. 6D). The associations between LINC00478 expression and immune cell infiltrations were further validated by CIBERSORT methods and shown in Figure S1. Taken together, these results suggest that LINC00478 modulates the infiltration of these immune cell subtypes in EC.

Discussion

The rapid development of molecular genomics has led to the identification of highly specific biomarkers for diagnosis and prognosis of cancer. This has provided a new molecular basis for improving the traditional diagnosis and treatment of cancer (Slack and Chinnaiyan 2019). lncRNAs have been shown to play vital roles in carcinogenesis (Profiling Noncoding RNAs in Cancer 2016). Dysregulation of lncRNAs has been strongly associated with tumor development and progression (Lin et al. 2020; Gong et al. 2022); therefore, it is indispensable to illuminate the function and underlying mechanisms of those novel lncRNAs. EC is one of the major types of gynecologic cancer affecting women's health. However, the mortality rate of EC has not been markedly improved due to the fact that the biological functions and molecular mechanisms involved in the occurrence and metastasis of EC remain largely unknown (Crosbie et al. 2022). In this study, we found that LINC00478 was significantly down-regulated in EC tissues compared with the corresponding non-tumor tissues. This was further validated by using TCGA and GEO data. Our results confirmed that LINC00478 was closely associated with the progression of EC and may be a potential therapeutic target for EC patients. Functionally, overexpression of LINC00478 significantly repressed the proliferation, clone formation, cell cycle, migration, invasion and tumorigenicity of EC cells *in vitro* and *in vivo*. Therefore, the present study demonstrated that LINC00478 may exert tumor suppressor effects on EC progression.

There are a large number of studies reporting that LINC00478 has been involved in the occurrence and development of multiple cancers, such as oral squamous cell carcinoma (Feng et al. 2017), vulvar squamous cell carcinoma (Ni et al. 2016) and bladder cancer (Yang et al. 2022). Therefore, LINC00478 has a great potential role in the prognosis and treatment targets of human cancer. Therefore, LINC00478 has a great potential role in the prognosis and treatment targets of human cancer. Our findings were consistent with previous reports. A study reported that LINC00478 expression was diminished in bladder cancer, and this lncRNA functioned anticancer effects in bladder cancer via the recruitment of histone demethylation transferase KDM1A to the MMP9 promoter region (Yang et al. 2022). Another evidence indicated that LINC00478 was differentially expressed in breast cancer, and further highlighted the diagnostic value of LINC00478 for breast cancer (Zhang et al. 2019). These clues provide a new scientific basis for the role of LINC00478 in human cancers. To further figure out the specific mechanism of LINC00478 acting as a cancer suppressor, we predicted the potential target genes of LINC00478 by bioinformatics tools. We found that LINC00478 could interact with PTBP1, which was confirmed by RNA pull-down and RIP assays. PTBP1 is involved in several steps in the metabolism of mRNAs, including mRNA stability, mRNA transport and internal ribosome entry site-mediated translation (Taniguchi et al. 2021).

Increasing evidence has substantiated PTBP1 exerts crucial roles in cell growth, migration, invasion, apoptosis and cell cycle, and is relevant with various malignancies (Wang et al. 2018; Wang et al. 2017; Caruso et al. 2017). One evidence showed that PTBP1 promoted the malignancy of ESCC cells by enhancing the IRES-mediated translation of cyclin B1 by binding to the 5'UTR of cyclin B1 (Fan et al. 2022). Another study indicated that PTBP1 participated in cancer metastasis by stabilizing CDCP1 mRNA (Cen et al. 2022). In this study, we found that the expression of PTBP1 was significantly induced in EC tissues, suggesting that PTBP1 acts as an oncogene in EC development. Notably, there was a negative correlation between LINC00478 and PTBP1 expression. Recently, cancer-specific energy metabolism, referred to as the Warburg effect, has been proposed as a cancer hallmark. Enhancement of the glycolysis pathway in cancer cells is what primarily characterizes the Warburg effect (Taniguchi et al. 2021; Vander Heiden et al. 2009). Previous studies have determined that PTBP1 is a crucial molecule for the maintenance of the Warburg effect in various types of cancer cells (Choksi et al. 2021). Therefore, we performed the experiment of glucose absorption and lactic acid production. Similarly, we observed that the EC cells with up-expressed LINC00478 together with low PTBP1 expression levels weakened glycolysis consumptions and

lactate productions, prompting that LINC00478 interacting with PTBP1 suppressed cell growth by impairing the Warburg effect in EC cells. Nevertheless, more detailed mechanisms of how LINC00478 modulating PTBP1 expression and related downstream molecules need to be investigated.

To further explore the molecular mechanism of LINC00478 in EC, we performed high-throughput sequencing to screen for candidate genes that were regulated by LINC00478. We identified thousands of differentially expressed genes between LINC00478 overexpression and control groups. Functional annotation analysis revealed that these genes were involved in various physiological functions and signaling pathways that were closely related to tumor progression. These findings may provide insights into the downstream targets and pathways of LINC00478 in EC. One of the important aspects of tumor progression is the interaction between tumor cells and the immune system. Tumor immunotherapy is a treatment method that harnesses the patient's own immune system to recognize and eliminate tumor cells, which has the advantages of high efficiency, durability and breadth (Sharma and Allison 2015). However, tumor cells can evade immune surveillance and attack by various mechanisms, such as down-regulating the expression of tumor antigens, inducing immune tolerance, secreting immunosuppressive factors and recruiting immunosuppressive cells (Chen and Mellman 2013). Therefore, understanding the molecular mechanisms of tumor immune interaction is crucial for developing effective immunotherapy strategies. LINC00478 may act as a potential target for tumor immunotherapy, as it modulates the infiltration and function of various immune cell subtypes in EC. As we mentioned before, LINC00478 expression was negatively correlated with some immune cell subtypes that have anti-tumor effects, such as activated CD4 T cells, activated CD8 T cells, activated dendritic cells, natural killer cells and type 1 T helper cells. These immune cell subtypes can directly kill tumor cells or secrete cytokines to activate other immune cells (Sharma and Allison 2015). Conversely, LINC00478 expression was positively correlated with effector memory CD4 T cells, which may have pro-tumor effects by promoting angiogenesis, invasion and metastasis (Gajewski et al. 2013). Moreover, LINC00478 expression was negatively associated with immune score and positively associated with stromal score, indicating that LINC00478 may reduce the immune activity and increase the stromal activity in EC tissues. These results suggest that LINC00478 may create an immunosuppressive microenvironment that favors tumor growth and progression.

However, these results seem to contradict the finding that LINC00478 is lowly expressed in EC and has a tumor suppressive role. How can we explain this paradox? One possible explanation is that LINC00478 may have different functions in different cellular contexts or at different stages of tumor development. For example, LINC00478 may act

as a tumor suppressor in normal or early-stage EC cells by regulating some cellular processes such as apoptosis, proliferation or DNA repair. However, when EC cells become more aggressive or resistant to therapy, they may down-regulate LINC00478 expression to escape from immune surveillance and attack. In this case, LINC00478 may act as an immunosuppressor in advanced or metastatic EC cells by modulating the infiltration and function of various immune cell subtypes. Another possible explanation is that LINC00478 may have different effects on different types of immune cells or different subsets of the same type of immune cells. For example, LINC00478 may inhibit some pro-tumor immune cells such as MDSCs or regulatory T cells, but also inhibit some anti-tumor immune cells such as natural killer cells or type 1 T helper cells. In this case, LINC00478 may have a complex and balanced role in regulating the tumor immune microenvironment in EC. Therefore, targeting LINC00478 may be a challenging but promising way to enhance the efficacy of tumor immunotherapy. Increasing LINC00478 expression may inhibit some pro-tumor immune cells and some anti-tumor immune cells in EC tissues, as well as inhibit the Warburg effect by down-regulating PTBP1 expression. The Warburg effect not only provides metabolic advantages for cancer cells, but also influences their interaction with the immune system (Ho et al. 2015). Therefore, targeting LINC00478 may be a challenging but promising way to enhance the efficacy of tumor immunotherapy. Increasing LINC00478 expression may inhibit some pro-tumor immune cells and some anti-tumor immune cells in EC tissues, as well as inhibit the Warburg effect by down-regulating PTBP1 expression. This may have a dual effect on the tumor immune interaction: On the one hand, it may reduce the immunosuppression and increase the immunostimulation; on the other hand, it may also reduce the metabolic advantages and increase the metabolic stress of tumor cells. The net outcome of this intervention may depend on the relative proportion and function of different immune cell subtypes and metabolic pathways in EC tissues. Alternatively, LINC00478 may be used as a biomarker to predict the response and prognosis of EC patients who receive immunotherapy.

Our study has some limitations that need to be addressed. First, we only used a medium-sized sample to analyze the expression levels of LINC00478 in EC tissues. More datasets from different sources are needed to validate our findings and increase their generalizability. Second, we only explored one potential target gene (PTBP1) of LINC00478 among several candidates predicted by bioinformatics tools. The functional roles and molecular mechanisms of other target genes remain to be elucidated. Third, we only demonstrated that LINC00478 could bind to PTBP1 protein by RNA pull-down and RIP assays. The exact binding sites and

domains between LINC00478 and PTBP1 need to be identified by further experiments. Fourth, we only evaluated the effects of LINC00478 on the proliferation, migration, invasion and tumorigenicity of EC cells in vitro and in vivo. The effects of LINC00478 on other biological processes, such as apoptosis, angiogenesis and immune response, are still unknown. Therefore, more comprehensive and in-depth studies are required to fully understand the role and mechanism of LINC00478 in EC progression.

In summary, we characterized LINC00478 as a tumor suppressor lncRNA that was down-regulated in EC tissues and could suppress tumor growth and metastasis of EC both in vitro and in vivo. Mechanistically, we demonstrated that LINC00478 could interact with PTBP1 to inhibit the Warburg effect in cancer cells, consequently restraining the EC development and progression. We hope our data shed light on the potential mechanisms related to lncRNA in the regulation of EC development, and further highlight LINC00478 as a promising therapeutic target for EC treatment.

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Author contributions FQ conceived and designed the study. YF and YD performed most of the assays. YY and WS help to perform the functional experiments. JZ, EZ and YC help to collect the samples and analyzed the data. YF, YD and BR wrote the first draft of the manuscript. SF, YZ, JL and FQ performed critical revision of the study. FQ confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

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

Declarations

Conflict of interest The authors declare no potential conflicts of interest.

Ethical approval and consent to participate The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Guangzhou Medical University, and written informed consent was obtained from all subjects. All experiments were conducted according to the relevant guidelines and following the approval of the Institutional Animal Care Committee of Guangzhou Medical University.

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