Article

J. Biol. Regul. Homeost. Agents. 2025; 39(1): 37–45
https://doi.org/10.23812/j.biol.regul.homeost.agents.20253901.5

The Transcription Factor ELK1 Promotes Esophageal Squamous Cell Carcinoma Progression by Activating the CDK4/Wnt/β-catenin Pathway

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Submitted: 17 October 2022 Revised: 18 January 2023 Accepted: 29 January 2023 Published: 16 January 2025

Objective: To explore the biological functions and the mechanisms of ETS transcription factor ELK1 (ELK1) in esophageal squamous cell carcinoma (ESCC) cells.

Methods: ELK1 expression in ESCC samples/cells was analyzed by the UALCAN database. ELK1 mRNA expression in ESCC tissue samples and cells was detected utilizing quantitative real-time PCR. Cell counting kit-8 method, BrdU assay and Transwell assays were employed for examining ESCC cell proliferation, migration, and invasion. The PROMO database was adopted to predict the binding sites of ELK1 and the cyclin-dependent kinase 4 (CDK4) promoter region. The binding status of ELK1 and CDK4 was predicted and verified by dual-luciferase reporter gene assay and chromatin co-immunoprecipitation qPCR assay. The effect of ELK1 on CDK4 expression was examined via western blot. The possibly related signaling pathways to CDK4 were predicted by the gene set enrichment analysis in the LinkedOmics database.

Results: In ESCC tissues and cells, ELK1 was up-regulated and was associated with short survival of patients. Upregulating ELK1 promoted malignant phenotypes of ESCC cells; ELK1 knockdown exerted the opposite effect. ELK1 bound to the CDK4 promoter region; ELK1 overexpression facilitated CDK4 expression, whereas ELK1 knockdown inhibited CDK4 expression. CDK4 is enriched in the Wnt signaling pathway, and ELK1 overexpression boosted β-catenin expression.

Conclusion: ELK1 promotes of CDK4 transcription, and activates Wnt/β-catenin pathway, thereby facilitating the ESCC malignant progression.

Keywords: ELK1; CDK4; ESCC; proliferation; migration; invasion

Introduction

Known as one of the most frequently occurring malignancies, esophageal carcinoma (EC) results in 480,000 new cases around the world annually [1]. The main pathological subtype of EC is esophageal squamous cell carcinoma (ESCC) [2]. The current therapeutic methods for ESCC include surgery, chemotherapy and radiotherapy, and despite continuous advances in these treatment means, most patients' 5-year survival rate remains less than 15% as a result of ESCC's high invasiveness, rapid progression, and being prone to drug resistance and recurrence [3,4]. In this context, seeking effective biomarkers is vital for future ESCC diagnosis and therapy.

Belonging to the ETS proto-oncogene family, ETS transcription factor ELK1 (ELK1) mainly contains four conserved domains: ETS DNA-binding domain at the N-terminal of ELK1 protein, B-box SRF-binding domain, transcriptional activation domain containing mitogen-activated protein kinase (MAPK) phosphorylation site, and MAPK anchor site [5,6]. Moreover, ELK1 is a regulator of the proto-oncogene c-fos, modulating cell differentiation, proliferation and apoptosis [7,8]. Reportedly, ELK1 plays a promoting part in the genesis of tumors such as bladder cancer (BC) [9], prostate cancer [10] and ovarian cancer [11]. Additionally, ELK1 is significantly high-expressed in cervical carcinoma tissues and cells, and ELK1 overexpression can boost tumor cell multiplication and invasion and restrain cell apoptosis and autophagy [12]. Although ELK1 has been proven to be high-expressed in ESCC tissues [13], the biological functions of ELK1 in ESCC and the molecular mechanisms remain unclear.

Cyclin-dependent kinase 4 (CDK4) is a kind of serine/threonine kinase that can bind to cyclin D to facilitate cell transition from G1 phase to S phase [14]. High CDK4 expression is strongly linked to poor prognosis of ESCC patients, and CDK4 overexpression facilitates ESCC cell multiplication and migration [15,16]. Furthermore, bioin-

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formatics prediction shows that CDK4 is a downstream target of ELK1. Therefore, this study is aimed at exploring the biological functions of ELK1 in ESCC by targeting CDK4 and the specific molecular mechanisms.

Materials and Methods

Collecting Clinical Cases

The 32 cases of ESCC and normal para-cancerous tissues were surgically removed in our hospital from October 2018 to March 2021. We froze the samples in liquid nitrogen within 30 min after they were removed and then stored them in the refrigerator. The ethical approval of the present study is not needed for the use of the human samples would not change the diagnosis and treatment processes of the patients, and the personal information of the patients would not be released during the study. But our work was still under the guidance of the hospital ethics committee, and performed according to the guidelines of *Declaration of Helsinki*.

Cell Culture

From the Cell Bank of the Chinese Academy of Sciences, we bought normal human esophageal epithelial cells (HEEC) and ESCC cell lines (KYSE-30, ECA-109, TE-1 and KYSE-150). Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), was used to culture the cells in the incubator at 37 °C under the conditions of 95% humidity and 5% CO_2. The origin of all of the cell lines used in this work was validated via STR method, and antibiotics were applied for preventing the contamination during the cell culture.

Cell Transfection

From RiboBio Co., Ltd., we purchased empty vector (NC), ELK1 overexpression plasmid (ELK1), CDK4 overexpression plasmid (CDK4), small interfering RNA (siRNA) targeting ELK1 (si-EKL1), targeting CDK4 (si-CDK4), and their negative controls (si-NC). Following the instructions of Lipofectamine® 2000 (Invitrogen), the transfection was carried out.

Quantitative Real-time PCR (qRT-PCR)

TRIzol reagent (Takara) was used to extract total RNA. Subsequently, 0.5 μg of total RNA was taken and reverse-transcribed into cDNA utilizing the Reverse Transcription Kit (Takara). Then, with the SYBR Green PCR Kit (Takara), qRT-PCR was performed: denaturation for 2 min at 94 °C, denaturation for 12 s at 94 °C, annealing for 12 s at 53 °C, and extension for 15 s at 72 °C, in 45 cycles. With GAPDH and U6 as the internal references, the relative expressions of ELK1 mRNA and CDK4 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. Primers:

ELK1, F: 5′- TCCCTGCTTCCTACGCATACA-3′, R: 5′-GCTGCCACTGGATGGAAACT-3′;

CDK4, F: 5′-TGACATTCCCCTCCCACCTCTCC-3′, R: 5′-ATCCTCCTGCCTCAGTCTCCCAAGTA-3′;

GAPDH, F: 5′-TGTTCGTCATGGGTGTGA AC-3′, R: 5′-ATGGCATGGACTGTGGTCAT-3′;

U6, F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′.

Cell Counting Kit-8 (CCK-8) Assay

About 2000 cells were added to each well. After culturing for 48 h, CCK-8 solution (20 μL/well, Dojindo) was added to continue the culturing for 4 h. Afterwards, the absorbance (OD) value at 450 nm of each well was detected through enzyme-linked immunosorbent assay to indicate the cell multiplication activity. The growth curve was drawn with the OD value as the y-coordinate and each time point of cell culture as the x-coordinate.

BrdU Assay

ESCC cells were grown in 24-well plates, and the medium was renewed after transfection to continue the culture. When the cells reached 80% confluency, 10 μmol/L BrdU (Beyotime) was added to the cells, and the plates were incubated for 4 h at 37 °C. After we discarded the cell culture medium, the cells were rinsed with PBS and added with 70% absolute ethanol, and then fixed for 10 min at 4 °C. Subsequently, we discarded the 70% absolute ethanol, and rinsed the cells with PBS three times, 5 min each time. The plates, after 2 mol/L HCl was added, were incubated for 40 min at 37 °C to denature cellular DNA. Then, we discarded the HCl, and washed the cells with PBS 3 times, 10 min each time. The plates, after 1.0% bovine serum albumin (BSA) was added to the cells, were kept for 1 h at room temperature for blocking. Subsequently, BrdU monoclonal antibody (ab6326, 1:300, Abcam) was added to the cells and incubated at 4 °C overnight. The next day, FITC-labeled Goat Anti-Rat IgG H&L (HRP) fluorescent secondary antibody (ab205720, 1:600, Abcam) was added, and they were incubated for 2 h at room temperature. After counterstaining the nuclei of all cells with PI, they were observed under the fluorescence microscope. We counted the number of BrdU-positive cells in 10 randomly selected non-overlapping fields ($\times$100), and calculated the mean value.

Transwell Assays

The 24-well plates were placed in the top compartment of the Transwell chamber (Applied Biosystems). Matrigel (not used in migration assay) was diluted with serum-free medium, and 50 μL was added to the upper compartment, and the chamber was kept for 4 h at 37 °C. ECA-109 and TE-1 cells during logarithmic growth were collected and cultured in serum-free medium for 12 h to prepare 2×10^5 cells/mL cell suspension. We then added 200 μL of cell suspension to the top chamber and 600 μL of 10% FBS-containing DMEM to the bottom chamber, and incubated the chamber at 37 °C for 48 h. Later, we took out the cham-

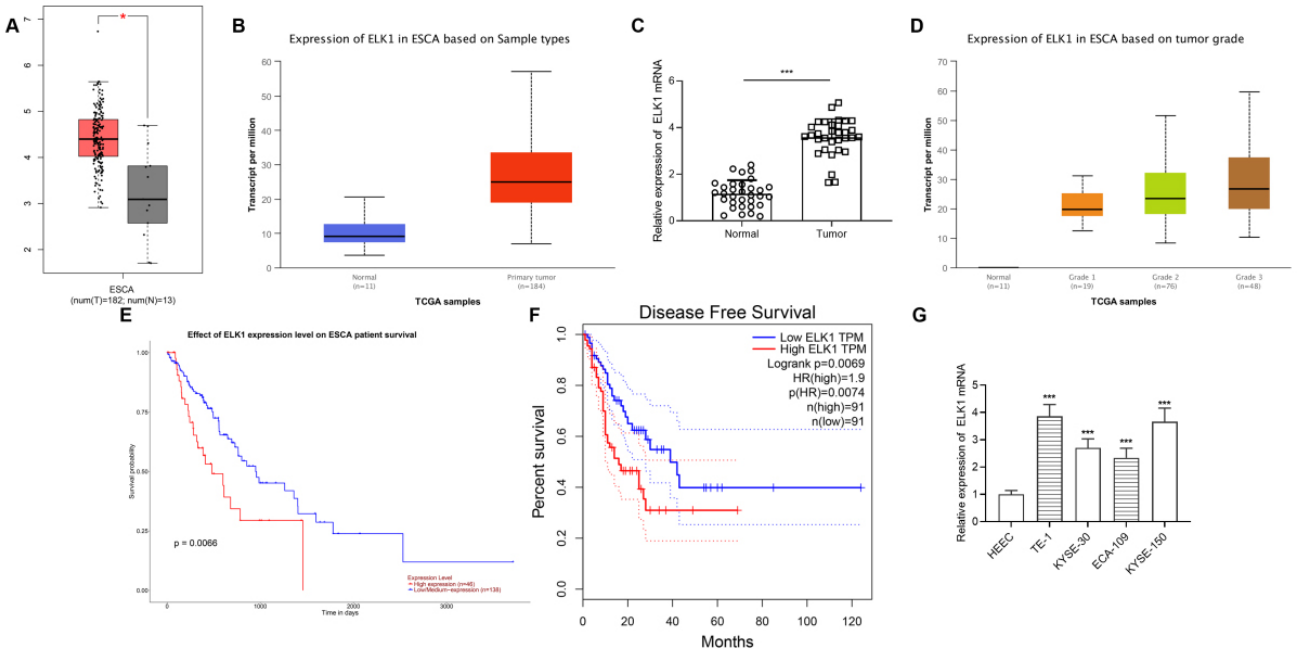

Fig. 1. ELK1 expression characteristics in ESCC. (A) ELK1 expression differences in ESCC and para-cancerous tissues (data from GEPIA database, *t* test). (B) ELK1 expression differences in ESCC and para-cancerous tissues (data from UALCAN database, *t* test). (C) ELK1 expression differences in 32 ESCC tissues and 32 normal tissues (qRT-PCR data, *t* test). (D) UALCAN database analysis of the relationship between ELK1 expression and tumor grade (one-way ANOVA with Tukey's post-hoc test). (E) The relationship between ELK1 expression and patient survival (data from UALCAN database, log-rank test). (F) The relationship between ELK1 expression and patients' DFS time (data from GEPIA database, log-rank test). (G) ELK1 mRNA expression in normal cells and ESCC cell lines (qRT-PCR data, one-way ANOVA with Tukey's post-hoc test). ELK1, ETS transcription factor ELK1; ESCC, esophageal squamous cell carcinoma; qRT-PCR, quantitative real-time PCR; DFS, disease-free survival.

ber, wiped off the cells failing to penetrate the Matrigel, stained the cells passing through the gel with 0.1% crystal violet, and counted their number under the microscope. The assay was repeated 3 times, and the cell invasion ability was expressed as the average value.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was conducted utilizing the EZ-ChIP™ kit (Millipore). After culturing ECA-109 and TE-1 cells for 36 h, they were fixed with formaldehyde. The fixation was terminated with glycine after incubation. The cells were scraped for obtaining cell pellets, and subsequently cell lysis buffer containing PMSF was added to obtain nuclear precipitates. After ultrasonic shearing of DNA in an ice bath, 10% of the volume of the supernatant in the ultrasonic shearing-resultant nuclear lysate functioned as the control, and the remaining 90% of the lysate was incubated with CDK4 antibody under magnetic bead shaking and centrifuged. Fresh elution buffer was used to elute the DNA bound to CDK4, and then a DNA purification kit was adopted to purify the DNA. Eventually, the purified DNA was detected through qRT-PCR.

Dual-luciferase Reporter Gene Assay

To construct the CDK4 wild-type promoter reporter gene vector (CDK4-WT) and the CDK4 mutant promoter reporter gene vector (CDK4-MUT), the wild-type or mutant CDK4 sequence was inserted into the pmirGLO reporter gene vector (Promega). HEK-293T cells were co-transfected with plasmid or siRNA and CDK4-WT or CDK4-MUT. The luciferase activity of HEK-293T cells was detected 24 h after transfection through the dual-luciferase reporter gene detection kit (Promega).

Western Blot Assay

TE-1 and ECA-109 cells in each transfection group were collected, added with RIPA lysis buffer (Beyotime), and placed on ice for 20 min. They were centrifuged at 13,000 r/min for 20 min at 4 °C, and a BCA assay kit (Peirce) was employed for determining the protein concentration of the supernatant. Next, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed, and subsequently proteins were transferred to the PVDF membrane. Next, the membrane, after being blocked with 2% BSA blocking buffer, was incubated overnight at 4 °C with the primary antibody recombinant Anti-CDK4 antibody (ab108357, 1:1000, Abcam). With GAPDH antibody

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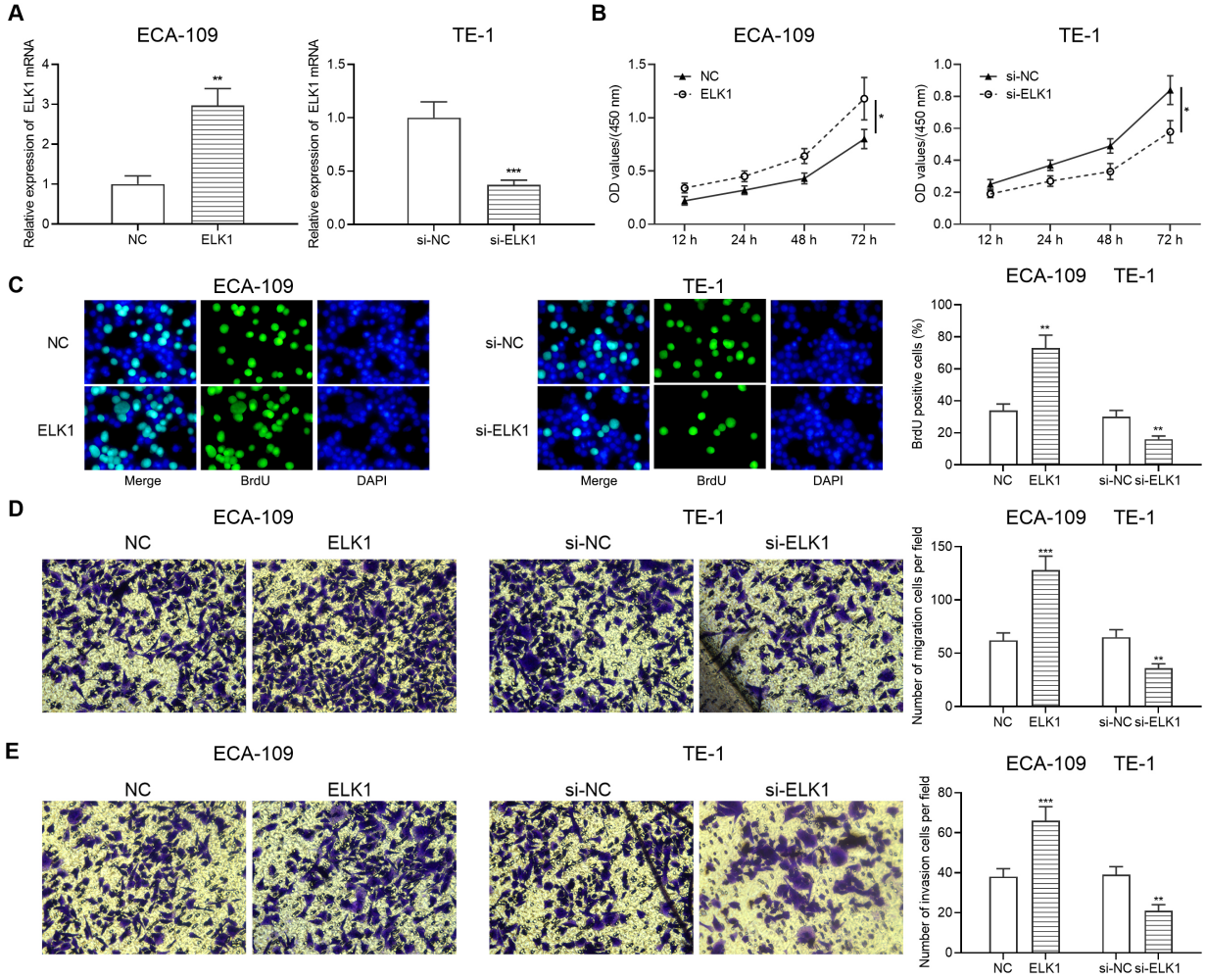

Fig. 2. Effects of ELK1 on ESCC cells. (A) ECA-109 cells were transfected with ELK1 overexpression plasmid or NC, and TE-1 cells were transfected with si-ELK1 or si-NC, and ELK1 mRNA expression was detected through qRT-PCR (*t* test). (B,C) After transfection, cell proliferation was examined by CCK-8 assay (B) and BrdU assay (C) (*t* test). (D) After transfection, Transwell assays were utilized for detecting cell migration and invasion (*t* test). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. NC, negative control; CCK-8, Cell Counting Kit-8.

as the reference, then the secondary antibody Goat Anti-Rabbit IgG H&L (ab97051, 1:2000, Abcam) was added, and they were incubated for 1 h at room temperature. Ultimately, the protein bands were developed employing the ECL kit (ThermoFisher Scientific), and the results were analyzed via Image GP software.

Statistical Analysis

SPSS 22.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) were utilized for statistical analysis. After normality test, the data were expressed as mean ± standard deviation. *T* test, and one-way analysis of variance with Tukey's post-hoc test were applied for data comparison. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were used to show the statistical significance.

Results

ELK1 is Markedly Upregulated in ESCC Tissues and Correlated with Poor Prognosis

By analyzing the GEPIA and UALCAN databases, it was unveiled that ELK1 is markedly upregulated in ESCC tissues as opposed to healthy tissues (Fig. 1A,B). In 32 cases of ESCC and matched healthy esophageal tissues, it was revealed that, ELK1 mRNA expression was elevated in ESCC tissues (Fig. 1C). Furthermore, the UALCAN database showed that high ELK1 expression is linked to increased tumor grade in ESCC (Fig. 1D). In addition, the UALCAN database and GEPIA database analysis manifested that high ELK1 expression is highly correlated to patients' short overall survival and disease-free survival (DFS) (Fig. 1E,F). Besides, ELK1 mRNA expression was also enhanced in ESCC cells (v.s. HEEC cells) (Fig. 1G).

A

Name	Dissimilarity	RE equally	RE query	Start	End	Sequence
Elk-1	0.0178	0.05608	0.05254	1073	1081	CTTCCTACT
Elk-1	0.0756	0.03204	0.02815	1254	1262	CTTCCGTCA

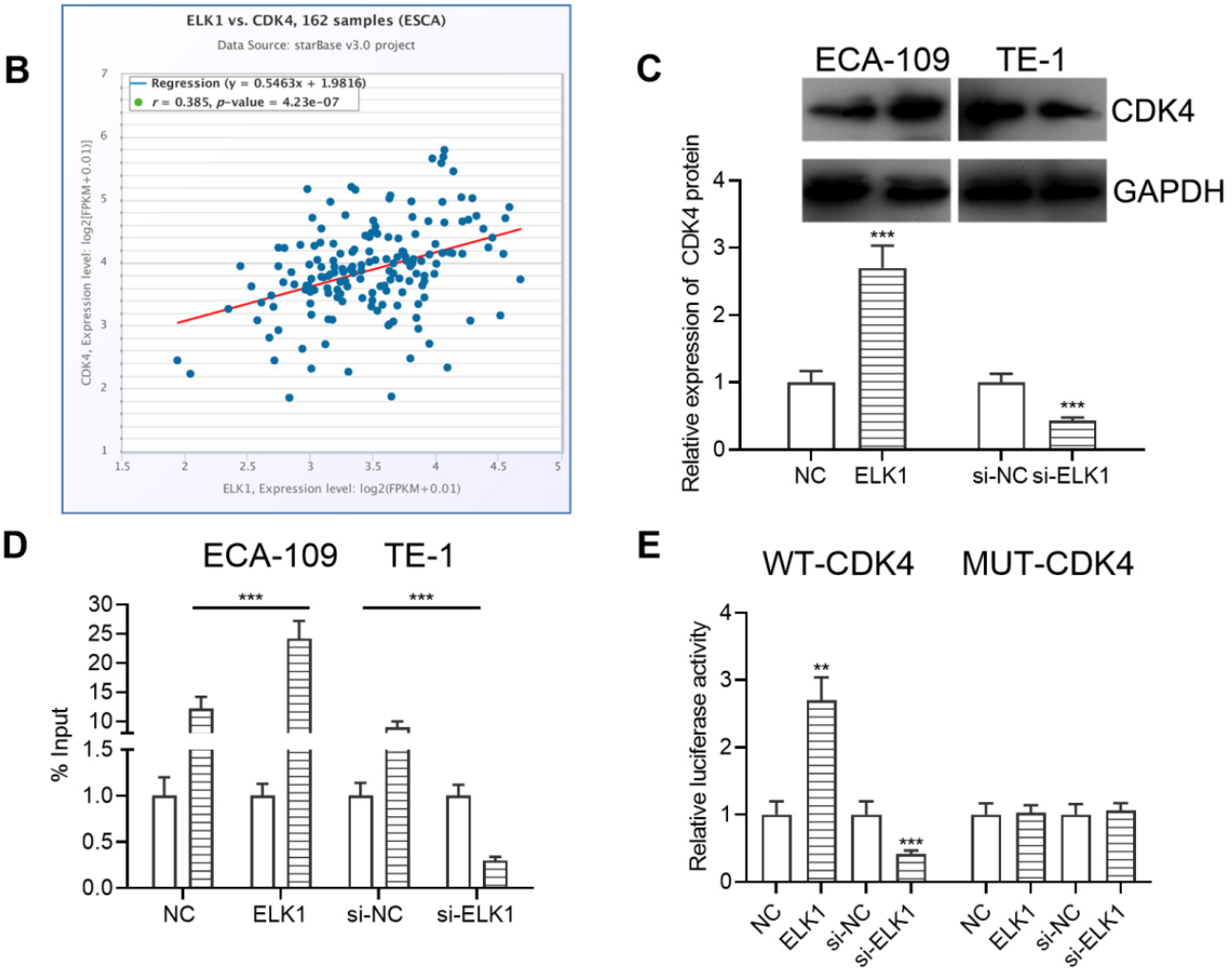

Fig. 3. ELK1 interacts with transcription factor CDK4. (A) Prediction of the binding sites of ELK1 to the CDK4 promoter region by the PROMO database. (B) The correlation between ELK1 and CDK4 expressions in ESCC tissues (data from StarBase database, Pearson's correlation test). (C) Western blot was employed to detect the effect of ELK1 overexpression or knockdown on CDK4 expression (*t* test). (D) ChIP-qPCR was conducted to examine the effect of ELK1 expression on the binding of ELK1 and CDK4 promoter regions (*t* test). (E) Effect of ELK1 expression on the luciferase activity of WT-CDK4 and MUT-CDK4 was verified via dual-luciferase reporter gene assay (*t* test). CDK4, cyclin-dependent kinase 4; ChIP, Chromatin Immunoprecipitation.

Effects of ELK1 on ESCC Cells

Then, ELK1 overexpression plasmids were transfected into ECA-109 cells, and si-ELK1 was transfected into TE-1 cells (Fig. 2A). As shown, up-regulating ELK1 promoted cell proliferation, whereas ELK1 knockdown restrained cell multiplication (Fig. 2B,C). Transwell assays showed that upregulating ELK1 markedly facilitated cell migration and invasion, yet ELK1 knockdown showed tumor-suppressive effect (Fig. 2D,E).

ELK1 Interacts with Transcription Factor CDK4

It was predicted that ELK1 can bind with the CDK4 promoter region by PROMO database (Fig. 3A). The StarBase database analysis manifested that ELK1 and CDK4 expressions are positively correlated in ESCC tissues (Fig. 3B). Western blot results manifested that ELK1 overexpression elevated CDK4 expression, while ELK1 knockdown lowered CDK4 expression (Fig. 3C). We speculated that ELK1 could boost the transcription of CDK4. To verify this regulatory effect, ChIP-qPCR assay was car-

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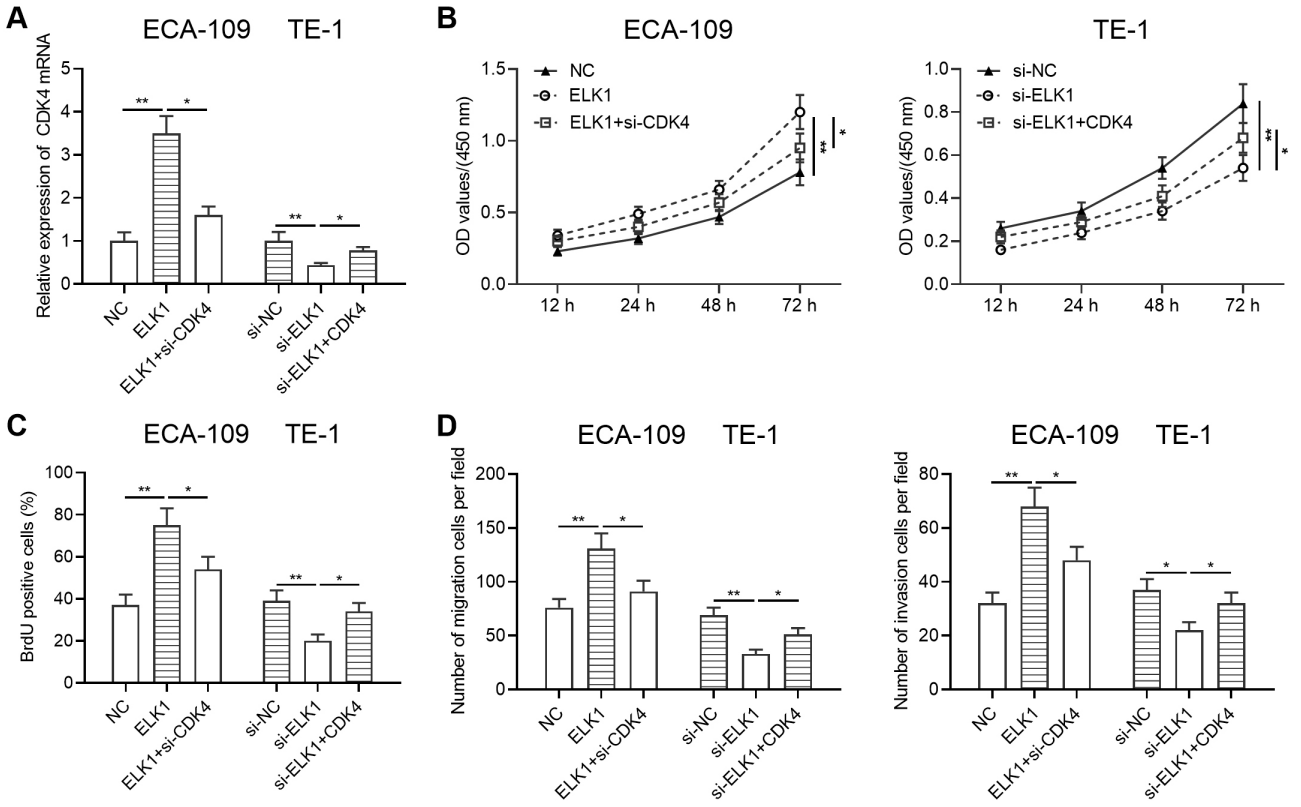

Fig. 4. ELK1's biological function is mediated by CDK4. (A) ELK1 overexpression plasmid and si-CDK4 were co-transfected into ECA-109 cells, and si-ELK1 and CDK4 overexpression plasmid were co-transfected into TE-1 cells; CDK4 mRNA expression was detected via qRT-PCR (*t* test). (B,C) After transfection, cell proliferation was detected by CCK-8 assay (B) and BrdU assay (C) (*t* test). (D) After transfection, cell migration and invasion were detected by Transwell assays (*t* test).

ried out using ELK1-specific antibodies to detect their binding relationship; the results suggested that, in contrast with NC, ELK1 overexpression increased their binding strength, and that, in contrast to si-NC, ELK1 knockdown decreased the binding strength (Fig. 3D). Consistently, it was revealed that ELK1 overexpression remarkably enhanced the luciferase activity of WT-CDK4, whereas ELK1 knockdown repressed that of WT-CDK4; neither overexpression or knockdown of ELK1 had a significant impact on that of MUT-CDK4 (Fig. 3E).

ELK1 Participates in Cell Proliferation, Migration, and Invasion through Targeting CDK4

To further confirm whether ELK1 promotes ESCC progression via modulating CDK4, ELK1 overexpression plasmid and si-CDK4 were co-transfected into ECA-109 cells, and TE-1 cells were co-transfected with si-ELK1 and CDK4 overexpression plasmid (Fig. 4A). As shown, ELK1 overexpression boosted cell multiplication, migration and invasion, whereas CDK4 knockdown could attenuate this effect; on the other hand, ELK1 knockdown could restrain cell proliferation, migration and invasion, whereas up-regulated CDK4 reversed this effect (Fig. 4B–D).

CDK4 Activates the Wnt Signaling Pathway and DNA Replication

Next, we performed gene set enrichment analysis (GSEA) employing the LinkedOmics database, and there was a possible association between high CDK4 expression and the activation of the Wnt signaling pathway and DNA replication (Fig. 5A,B). Next, the effects of ELK1 and CDK4 on β-catenin protein level were examined by western blot, and ELK1 overexpression facilitated β-catenin expression, yet CDK4 knockdown attenuated this effect; ELK1 knockdown suppressed β-catenin expression, while upregulating CDK4 counteracted the above-mentioned effect (Fig. 5C,D).

Discussion

ETS transcription factors are one of the largest transcriptional regulator families in cells, and the family members contain two domains: a transcriptional activation domain and a DNA-binding domain [17]. The DNA-binding domain is highly conserved, and it can bind with specific DNA sequences to modulate transcription, and further participate in many pathological and physiological processes via regulating cell differentiation, prolifera-

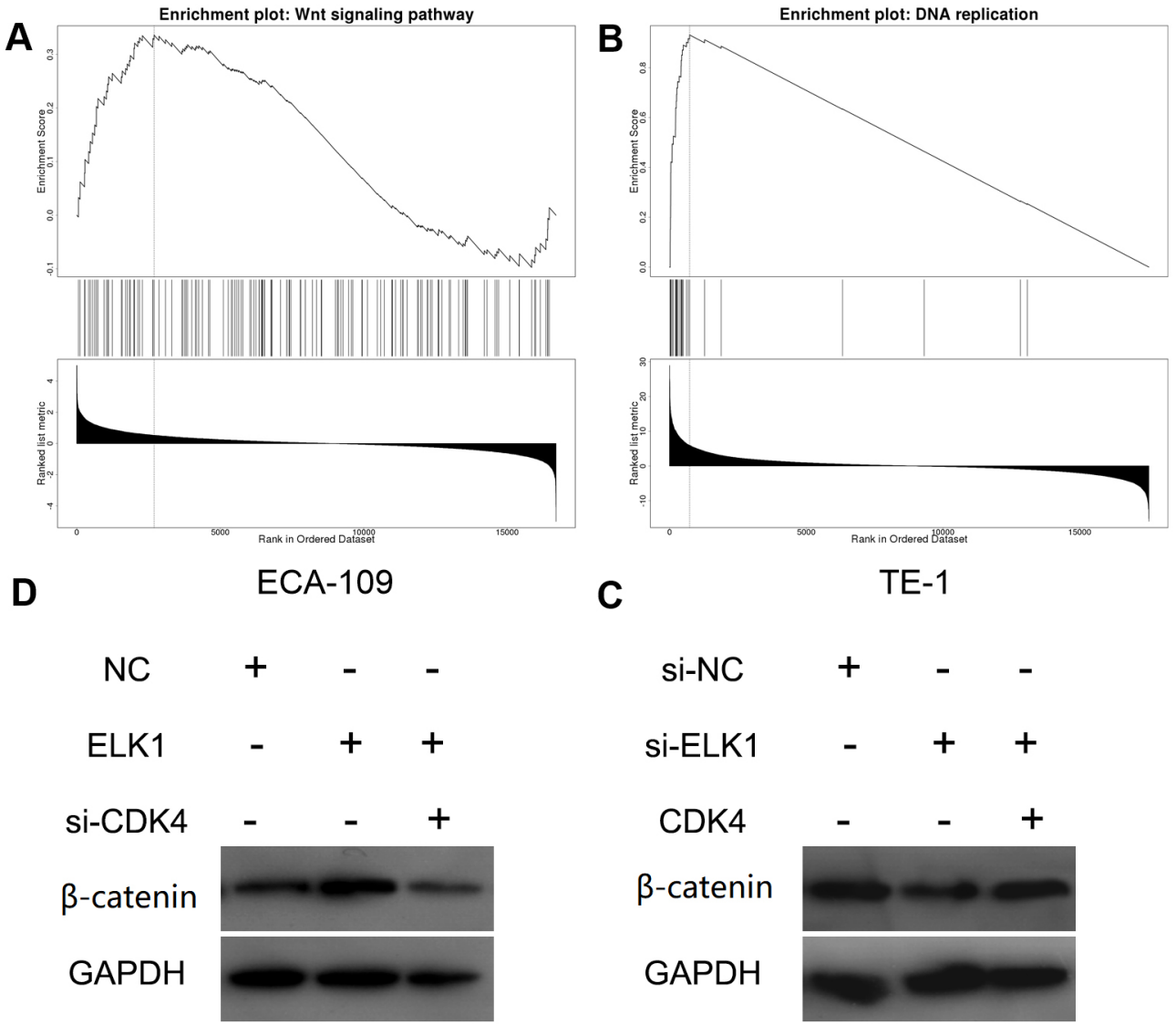

Fig. 5. CDK4 activates the Wnt signal pathway and the DNA replication pathway. (A,B) Signal pathway enrichment analysis using GSEA. (C,D) ELK1 overexpression plasmid and si-CDK4 were co-transfected into ECA-109 cells, and si-ELK1 and CDK4 overexpression plasmid were co-transfected into TE-1 cells; β-catenin expression was analyzed by Western blot.

tion, apoptosis, and other processes [18–20]. Various external stimuli phosphorylate ELK1, as a member of the ETS family, through the MAPK signaling pathway, causing ELK1 conformational changes, thereby enhancing its DNA-binding activity, activating transcriptional activity, upregulating growth and differentiation-related genes, inhibiting apoptosis-related genes, and ultimately partaking in the genesis and development of various malignancies [19,21]. For instance, ELK1 is observably overexpressed in thyroid cancer tissues and cells, and ELK1 knockdown can promote Egr-1 and PTEN expressions [22]. ELK1 expression is noticeably enhanced in BC, and is positively modulated by lncRNA-SNHG7 [23]. ELK1 is dramatically high-expressed in pancreatic carcinoma cells and tissues, and ELK1 overexpression can counteract the tumor-suppressive effects of miR-597-5p [24]. In ESCC, research has manifested that, in comparison to normal esophageal epithelium, the transcription factor ELK1 expression is markedly increased in ESCC tissues [13], but further biological functions and molecular mechanisms have not been studied for the time being. Herein, the dysregulation of ELK1 in ESCC was further validated, and its role in predicting patients' prognosis is preliminarily unveiled. Moreover, ELK1 overexpression boosted ESCC cell multiplication, migration, and invasion, whereas ELK1 knockdown resulted in the opposite impact. Based on our findings, it was confirmed that ELK1 plays a cancer-facilitating part in ESCC.

Cyclin-dependent kinases (CDKs) are proteins with serine/threonine protein kinase activity, which are the core part of the cell cycle regulatory mechanism, and have long

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been considered an important target in the treatment of malignancies [25,26]. CDK4 is a key protein in the cell cycle process and has the dual role of initiating DNA replication and promoting cell mitosis; its overexpression can accelerate the process of S phase and make cells excessively proliferate [27]. Furthermore, abnormal expression of CDK4 is linked to tumorigenesis [28–30]. In EC tissues and cells, YAP1 overexpression can positively regulate CDK6 expression, thus boosting the radiation resistance of EC, and CDK4 inhibitor treatment can make EC cells sensitive to radiation [31]. In ESCC tissues and cells, CDK4 is dramatically upregulated, and CDK4 is negatively regulated by miR-1, thus suppressing ESCC cell proliferation and facilitating the apoptosis [32]. Another study shows that the CDK4 inhibitor SHR6390 can restrain phosphorylated Rb and arrest the cell cycle in G1 phase, exerting antitumor activity in ESCC [33]. We found that ELK1 could bind with the CDK4 promoter region to activate the transcription of CDK4, partly explaining the mechanism of CDK4 dysregulation in ESCC. Wnt/β-catenin modulates multiple biological behaviors of ESCC cells. Specifically, a recently published work reports that PCDH20 represses ESCC progression via inactivating β-catenin [34]. Additionally, targeting integrin-linked kinase is revealed to sensitize ESCC cells to cisplatin, which is mediated by inhibition of Wnt/β-catenin pathway [35]. Importantly, it is also reported that CDK4 interacts with Wnt/β-catenin pathway. cyclin D2-CDK4/6 complex is required for Wnt/β-catenin-mediated intestinal hyperproliferation [36]. However, the interaction between CDK4 and Wnt/β-catenin in cancer biology is unknown. Here our data indicated that the ELK1/CDK4 axis could activate Wnt/β-catenin pathway, thereby playing a tumor-promoting role in ESCC.

To sum up, in ESCC ELK1 is upregulated, and its high expression is strongly linked to ESCC patients' poor prognosis. In terms of mechanism, ELK1 activates Wnt/β-catenin signaling by modulating CDK4, thereby facilitating the malignant phenotypes of ESCC cells.

Availability of Data and Materials

Not applicable.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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